Development of Antiviral Therapy against Hepatitis E Virus Infection

On the Basis of Host Factors

Yijin Wang

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Frequently used abbreviations

BQR	Brequinar
CypA/B	Cyclophilins A/B
COX1	Cytochrome c oxidase subunit I
ddC	2’, 3’-dideoxycytidine
DMSO	Dimethyl sulfoxide
Drp1	Dynamin-related protein 1
EB	Ethidium bromide
HEV	Hepatitis E virus
IMPDH	IMP dehydrogenase
ISGs	Interferon-stimulated genes
ISRE	Interferon-stimulated response element
FK506	Tacrolimus
LFM	leflunomide
MAVS	Mitochondrial antiviral signaling protein
Mff	Mitochondrial fission factor
Mfn1	Mitofusin-1
MPA	Mycophenolic acid
mtDNA	Mitochondrial DNA
MTND1	NADH-ubiquinone oxidoreductase subunit 1
MTX	methotrexate
NSP	Non-structure protein
OPA1	Optic atrophy 1
PKC	Protein kinase C
PMA	Phorbol 12-myristate 13-acetate

POLG	mtDNA polymerases γ
RBV	Ribavirin
RdRp	RNA dependent RNA polymerase
6-AU	6-Azauracil
6-TG	6-Thioguanine

CHAPTER 1

General Introduction and Outline of the Thesis

Hepatitis E virus is an important target for experimental virology

Hepatitis E virus is an emerging health issue world-wide and its morbidity is on the rise. HEV was isolated and discovered in faecal extracts from infected soldiers during the Soviet occupation of Afghanistan in the 1980s. For a long time, HEV was considered to be a significant public health problem but confined to developing countries with poor sanitary conditions. Now, however, it is recognized to be prevalent in industrialized countries as well [1-5]. As one of the most common causes of acute viral hepatitis, it provokes approximately twenty million HEV infections and three million HEV cases per year. There are also around 70 thousand deaths associated with HEV infection annually [6]. Although only a single HEV serotype exists, four genotypes of human HEV have been identified, genotype 1 and 2 are restricted to human beings, primarily found in developing countries and are transmitted via the fecal-oral route (involving *e.g.* contaminated water sources), and exhibits a mortality rate from 0.5% to 3% in young adults [7]. In contrast, the potentially zoonotically-derived genotypes 3 and 4 are prevalent in industrialized countries, with sporadic occurrence and spread mainly through eating undercooked pork or game products [8]. HEV is originally considered to be a self-limited, acute disease associated with low mortality, however, the main clinical challenge is posed by HEV genotype 3 infection in patients receiving orthotopic organ transplantation. More than 60% of organ recipients infected with HEV will develop chronic hepatitis with rapid progression to cirrhosis [8-10]. The mortality in pregnant women with acutely infection by genotype1 HEV was described to reach on average a rate of 25% [11-13]. Hence HEV represents an important target for investigation in current experimental virology.

The molecular virology of HEV is still only partially understood

HEV was originally classified as a non-A, non-B hepatitis. With the evidence emerging that disease associated with the detection of a highly conserved RNA-dependent-RNA-polymerase (RdRp) in a cDNA library constructed from infectious bile, the existence of a RNA virus was inferred that would constitute the pathogen for this non-A, non-B hepatitis and thus HEV was defined [14]. Subsequent investigations on this virus belonging to the family *Hepeviridae* showed that HEV is a non-enveloped single positive-stand RNA virus with a size of 27-34nm [15]. The genome is approximately 7.2-kb in size. It starts with a short 5′

noncoding region (NCR), followed by three open reading frames (Figure 1). ORF1 of HEV is the largest ORF in nucleotide length [16,17], encoding the nonstructural proteins of the virus and contains several functional domains, including a methyltransferase, a protease, a RNA helicase, and a RNA-dependent RNA polymerase, all involved in viral replication and protein processing [18, 19]. This ORF starts at the 5' end of the genome after the 25 bp NCR and can be translated directly from the HEV genome following infection. A hypervariable region (HVR) that distinguishes different HEV strains and genotypes with respect to the characteristics of viral replication and their differences in *in vitro* infection efficiency is also present in ORF1 [20]. The question whether the ORF1 product functions as a signal polyprotein or needs to be cleaved to smaller molecules by viral or cellular proteases is still controversial [21-24]. The majority of studies, however, support the later notion and suggest that HEV ORF1 is typically processed into small units, each exerting specific functions. The products of ORF2 are responsible for the immunogenicity of HEV and encode the capsid proteins necessary for virion assembly exploiting the host cell cytoplasmic machinery. Several host proteins are implicated in supporting the HEV life cycle, especially HSP90, Grp78 and HSPGs, which play a role in facilitating HEV entry into the host cells and its subsequent intracellular transport [25]. Obviously, in the HEV life cycle the capsid protein needs to combine with HEV genomic RNA for infectious viral particle formation [26]. Intriguingly, however, the capsid protein was also found to interact with cellular proteins involved in cellular signaling, potentially provoking inhibition of apoptosis and inhibition of NF- κB activation, which may aid HEV replication and infection by counteracting programmed cell death of infected cells and by constraining cell-autonomous immunity [27]. ORF3 encodes multifunctional proteins that are responsible for virion morphogenesis and release, but not replication [28, 29]. ORF2 overlaps with ORF3 and there is a junction region (JR) between ORF1 and the subgenomic coding region [30]. Additionally, two cis-reactive elements (CREs) overlap with ORF2 and the JR, respectively [31]. Both of the CREs are essential for HEV replication [31]. The 5′ end of the HEV genome contains a 7-methylguanosine cap structure, which is essential for HEV infection. Although ORF3 is not fully characterized, many studies demonstrated that ORF3 plays multiple roles during HEV infection by activating or inhibiting specific cellular signaling pathways. For example, ORF3 can modulate host gene expression by binding and activating mitogen-activated protein kinase (MAPK), a cardinal regulator of cellular gene expression [32]. ORF3 can also downregulate STAT3-mediated gene expression, but concomitantly enhance host

interferon induction through increasing RIG-1 expression [33, 34]. Interestingly, OPR3 has also been identified as a protective factor with respect to from mitochondrial depolarization and demise by stimulating mitochondrial voltage-dependent anion channel genes, indicating that ORF3 plays an important role in inhibiting mitochondrial apoptosis pathways [35].

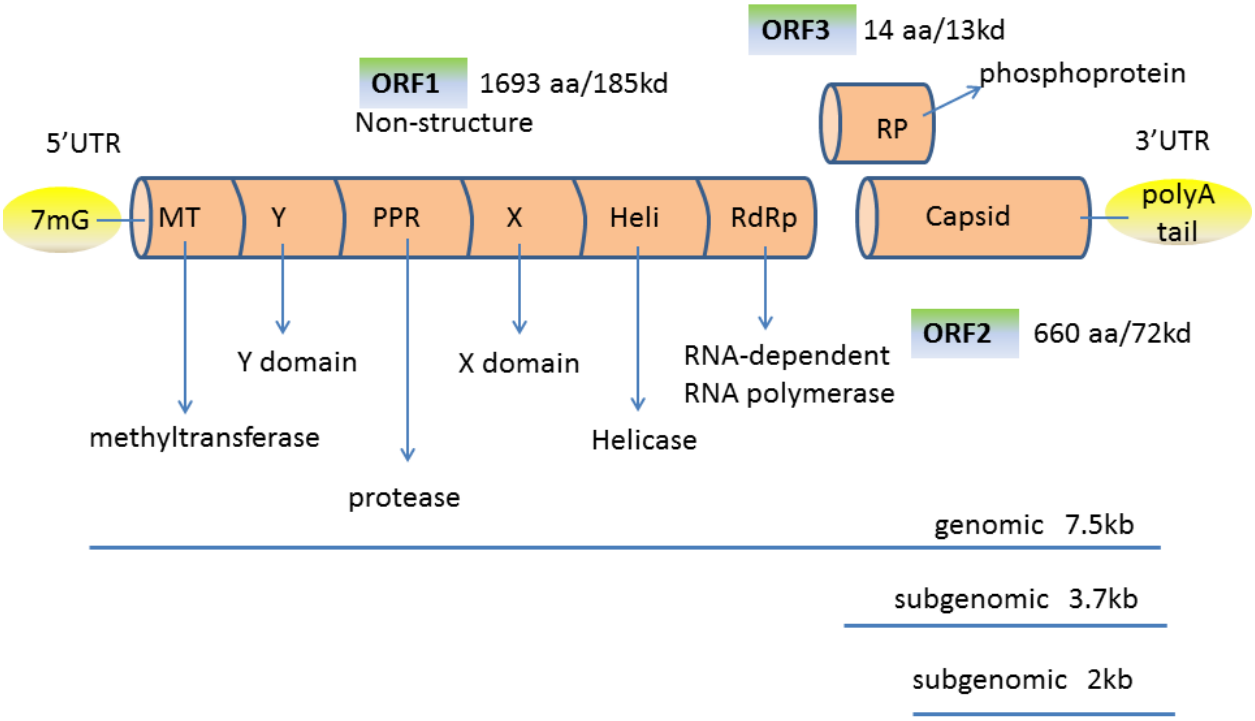

Figure 1. Overview of HEV RNA genome. The genome is 7.2-kb in length. It starts with a short 5'noncoding region (NCR), followed by three open reading frames (ORF). ORF1 encodes a polyprotein that gives rise to viral nonstructural proteins and is created as a 1693 amino acid (aa) precursor. From it a methyltransferase (MT) derived, a putative papain-like cysteine protease (PPR), RNA helicase (Heli), RNA-dependent RNA polymerase (RdRp) and a hypervariable region (HVR) are derived. ORF2 encodes a capsid protein of 660 aa. ORF3 encodes a small multifunctional protein of 14 aa.

The life cycle of HEV is relatively poorly understood. A putative HEV infection cycle has been proposed and is depicted in Figure 2. The entry of HEV into the cell is initiated by ORF2 capsid protein binding to cellular surface receptors [36]. However, the exact cellular receptor for HEV remains still unidentified despite several studies proposing putative receptor proteins on the cell or candidate binding sites located in HEV, and thus requires further elucidation [37-39]. Subsequently, the RNA genome of virus becomes uncoated and is subject to intracellular transport as to allow translation of the three ORFs. The translation of non-structural proteins in ORF1 yields RdRp, necessary for the production of negative-sense RNA, which serves as a template for positive-strand RNA formation and thus for viral progeny.

Then the ORF2 and ORF3 subgenomic RNA are translated into capsid proteins. The non-structural proteins subsequently package genomic viral RNA to assemble new virion [40]. ORF3 plays a crucial role at last step of viral egress by contacting with cell membrane. Thus although significant progress has been made, HEV biology remains only partially elucidated, prompting further investigations.

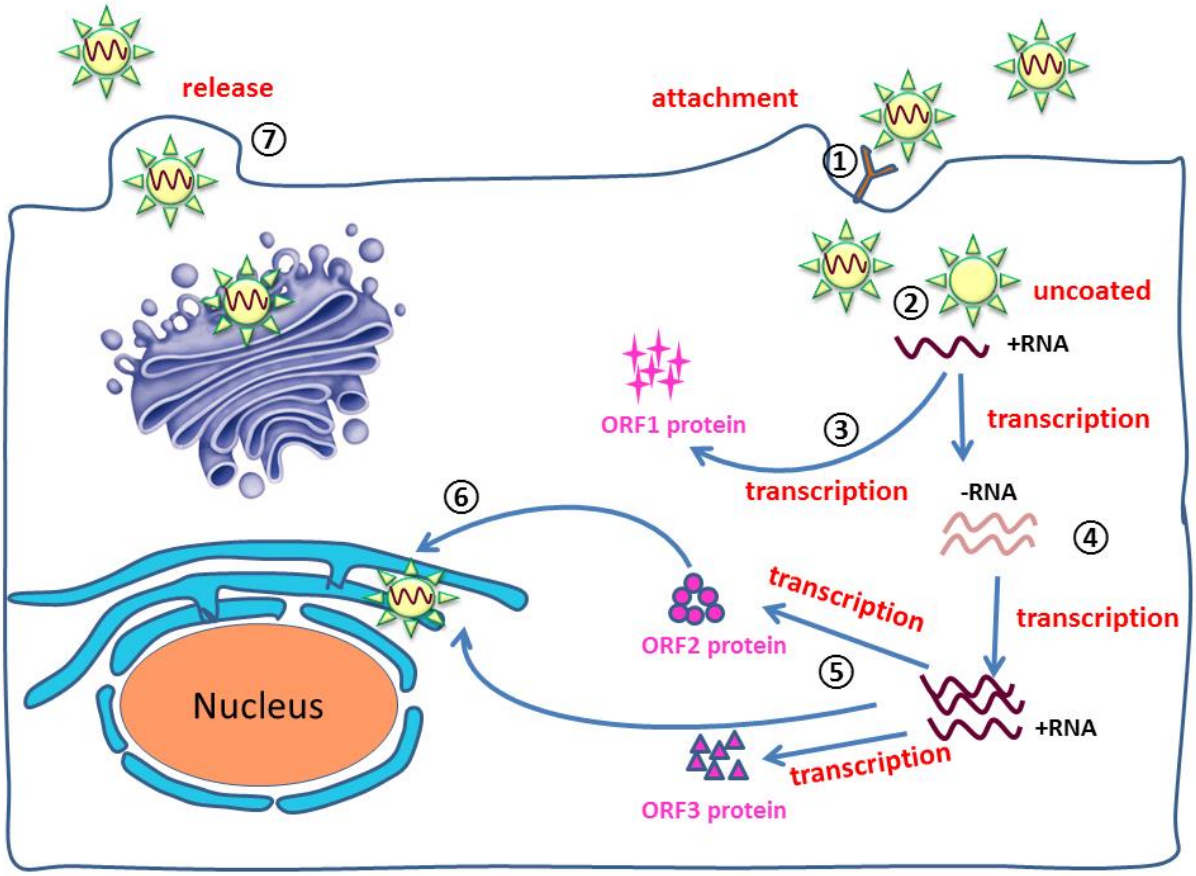

Figure 2. Life cycle of HEV. (1) Viral attachment to cell surface and entry through receptors; (2) Virus genome is uncoated and positive-strand RNA is released into the cytosol; (3) Translation of viral RNA to ORF1 non-structure protein; (4) Replication of viral genome RNA with negative-sense RNA being an intermediate, by the action of RdRp; (5) Translation of the subgenomic RNA located in ORF2 and ORF3; (6) Packaging of full-length genomic RNA into ORF2-derived capsid proteins and their assembly into presumptive virion; (7) Transport of the newly formed virion by ORF3 to the cell membrane and its release from the infected cells.

The lack progress in understanding HEV biology could largely be attributed to absence of stable HEV cell culture models, which had hampered investigation of HEV for a long time. Recently, however, the situation has dramatically improved with the development of a genotype 3-based cell culture system that provides a bona-fide and efficient tool for HEV research [41, 42]. I exploited this situation in the research described in this thesis. The data

obtained in my thesis are mostly on the basis of the genotype 3 infected HEV replication and infection cell cultures. A plasmid construct containing the full-length HEV genome (Kernow-C1 p6 clone, GenBank Accession Number JQ679013) and a construct containing subgenomic HEV sequence coupled with a Gaussia luciferase reporter gene (p6-luc) were used to generate HEV genomic RNA by using the Ambion mMESSAGE mMACHINE *in vitro* RNA transcription Kit (Life Technologies Corporation, Carlsbad, CA). Huh7 cells were electroporated with p6 full-length HEV RNA or p6-Luc subgenomic RNA to generate infectious or replication models, respectively. The contemporary body of biomedical literature indicates that this constituted a valid strategy for investigating the biology of HEV and it allowed me to investigate the role of host factors as targets for anti-viral therapy.

Acute and chronic infection of HEV – outstanding questions

HEV is one of the most common causes of acute hepatitis. Although the most common transmission routes of HEV have been identified, and appear to include contaminated food, contaminated water, uncooked meat, in most cases source of infection remains uncertain. In majority of immunocompetent patients, the virus manifests itself as an acute HEV infection with self-limiting and low motility with respect to inter-individual transmission (2%) properties, and is characterized by full recovery within 4-6 weeks following virus exposure [43]. An evident risk population for HEV acute infection accompanied by severe symptoms are pregnant women in India [13, 44, 45]. The mortality in this population reaches up to 25% and is largely restricted to HEV genotype 1 and 2-mediated disease, even though a few case studies reported infection with genotype 3 and 4. Despite of the finding that HEV viral load was evidently higher in pregnant female as compared with non-pregnant patients, further studies are needed to clarify the underlying cause of the excessive mortality rate in pregnant female with HEV infection. In addition further understanding of the biology of HEV will aid the rational design of strategies aimed at reducing mortality in this group of patients.

Chronic HEV infection is defined as persistent detection of HEV replication, lasting at least 3-6 months after acute infection became evident. Of note, and referring only to a limited number of reports, only genotype 3 appears capable to progress to chronic HEV infection, while genotype 1,2 and 4 of HEV have solely been described in the context of acute infection [46]. Chronic HEV infection that strikingly and rapidly progresses to fibrosis and

cirrhosis becomes increasingly documented in immunocompromised patients, especially in recipients of solid organ transplantation receiving immunosuppressive therapy but also cases of HIV co-infected patients have been described [9, 10, 47, 48]. The course of liver fibrosis course in chronic HEV-infected solid organ transplant recipients appears to be more severe than that observed in solid organ transplant patients with chronic HCV infection, as evident by progression full-blown cirrhosis 2-3 years following initial HEV infection [49]. No correlation has been observed between HEV load and the progression of liver fibrosis [50]. Studies show that the lower adaptive immune response in organ transplant patients with immunosuppressants therapy is likely the key factor explaining the sensitivity to and the clinical of chronic HEV infection in organ orthotopic transplantation recipients [9, 51-56]. The patients with persisting HEV infection presented evidently reduced size of the CD2, CD3 and CD4 T-cell compartment as compared to those that cleared HEV spontaneously [9]. Clinical analysis of the association between various of immunosuppressive medicines with the development of chronic HEV infection revealed that tacrolimus are associated with a worse disease course as compared to those patients using other immunosuppressants, especially cyclosporine [54] . Mechanistically the interaction of different immunosuppressants and HEV lifecycle remains obscure at best, however, prompting further investigations.

Cases of chronic HEV infection have also been observed in hematological patients and patients with pre-existing liver disease [57-59]. The prevalence or incidence of this chronic HEV infection manifestation has not been recorded systematically, because of the small amount of clinical cases involved.

Current treatment for HEV – room for improvement

In lieu of approved medication, only a few acute HEV infection patients have been given antiviral treatment, except in those instances when patients were considered to be severely ill and at high risk, such as those with pre-existing liver disease. This experience has suggested that monotherapy with ribavirin is sufficient for rapid clearance of HEV [60, 61].

The development of HEV infection has been studied mainly in organ transplantation patients with chronic HEV infection because they are administered with immunosuppressants for preventing organ rejection, which has been proposed to be a key factor for developing chronic hepatitis after HEV infection. Thus, reduction of

immunosuppressive therapy is the first line of strategy for combatting chronic HEV infection, which is sufficient in approximately one third of patients for clearance of the virus [54]. In case of non-response to the reduction immunosuppressants [62] or those cases that cannot tolerate a reduced dose of immunosuppressive drugs, pegylated interferon (peg-IFN), ribavirin monotherapy or a combination have been used as antiviral therapy, and have achieved HEV clearance in the majority of patients, despite that both of the medicines are not FDA-approved anti-HEV drugs. It is the opinion of the author of this thesis ribavirin monotherapy has now become the most prevalent treatment modality in this respect, probably because of its apparently validated efficiency towards chronic HEV infection in large case series reports [63]. A very recent study showed that ribavirin treatment failure for chronic HEV was the consequence of a specific mutation in specific HEV polymerase, providing an intriguing insight into the factors that underlie ribavirin resistance, while simultaneously highlighting the need for novel therapeutic modalities to combat HEV infection [64].In this context it is important to note that peg-IFN monotherapy for kidney, heart, or long transplant recipients must be done with caution because of the associated high risk for acute rejection [65, 66]. Nevertheless, also in view that all observations are based on the scattered case reports or small case series and thus that firm conclusions still require systematic analysis like large cohort evaluation or randomized trials [67], the conclusion is justified that clinical management of HEV still lacks appropriate medication.

In this context, one should take into account that different from patients with chronic HEV infection, fulminant HEV-induced hepatitis is a major concern, especially genotype 1 strains in pregnant women with their associated high mortality rates requiring improved treatment modalities. To date no evidence is available on the role of pharmaceutical intervention in the treatment in pregnant women with acute hepatitis E, non-pregnant patients with acute liver failure, or during neonatal HEV infection. The largest challenge in this respect is arguably hitting the temporal therapeutic window, when curative treatment might still be possible [67].

Manipulation of Immunosuppressant use in HEV infection

Immunosuppressants are used life-long in organ transplant patients in order to prevent rejection, but by definition put patients at risk for becoming immunocompromised.

Thus it is not surprising that administration of immunosuppressants also affects host immunity against viral challenges, putting patients at risk for development of hepatitis infection. Nevertheless, different immunosuppressive regimen may have very different effects on viral susceptibility and this aspect deserves urgent investigation.

In agreement with the above-mentioned notion, the majority of chronic HEV patients are derived from the organ transplantation recipient population, who receive immunosuppressants for preventing organ rejection, resulting in immunosuppression. Furthermore, good evidence exists that chronic HEV infection was diagnosed and progressed to cirrhosis usually in immunosuppressed individuals associated with weak adaptive immune response [49, 68]. However, clinical evidence obtained hitherto suggests not all the immunosuppressants facilitate HEV infection, as might be expected, but that different immunosuppressive regimens can differentially affect the infection course of HEV. The calcineurin inhibitor tacrolimus, but not cyclosporin A (CsA), has been found to be more frequently associated with persistent HEV infection [54], and mycophenolate mofetil, the pre-drug form of mycophenolic acid (MPA) can help to clear the virus [56]. However, due to insufficient cohort clinical data and obscurity at to the mechanistic insight with respect to which key factors determine chronic HEV infection course and how such factors are influenced by different immunosuppressants, the association of various of immunosuppressive medications and outcomes of HEV infections is still uncertain.

Fortunately, with the establishment of robust genotype 3 HEV cell culture models I was able to evaluate the distinct impacts of various of immunosuppressive agents on HEV replication and infection *in vitro*. In **chapter 3 and chapter 4**, I describe how different immunosuppressants affect HEV infection in cell culture models and prove that different immunosuppressants interact radically different with the HEV life cycle.

De novo nucleotide synthesis as an anti-viral target

Understanding virus and host interaction is essential for development of anti-viral medicine. The purine and pyrimidine nucleotides play critical role in cell metabolism such as genome replication and replication through producing ATP, GTP, UTP and CTP. Ribonucleotides can be synthesized through *de novo* pathways, which involve their assembly starting from fairly simple small molecules and proceeded piece-by-piece to end

products, each step catalyzed by specific enzymes, culminating in the attachment of the product to ribose (Figure 3). Alternatively, nucleosides can also be supplied by salvage pathways, which convert residual purine and pyrimidine bases into nucleotides by reconnecting to ribose.

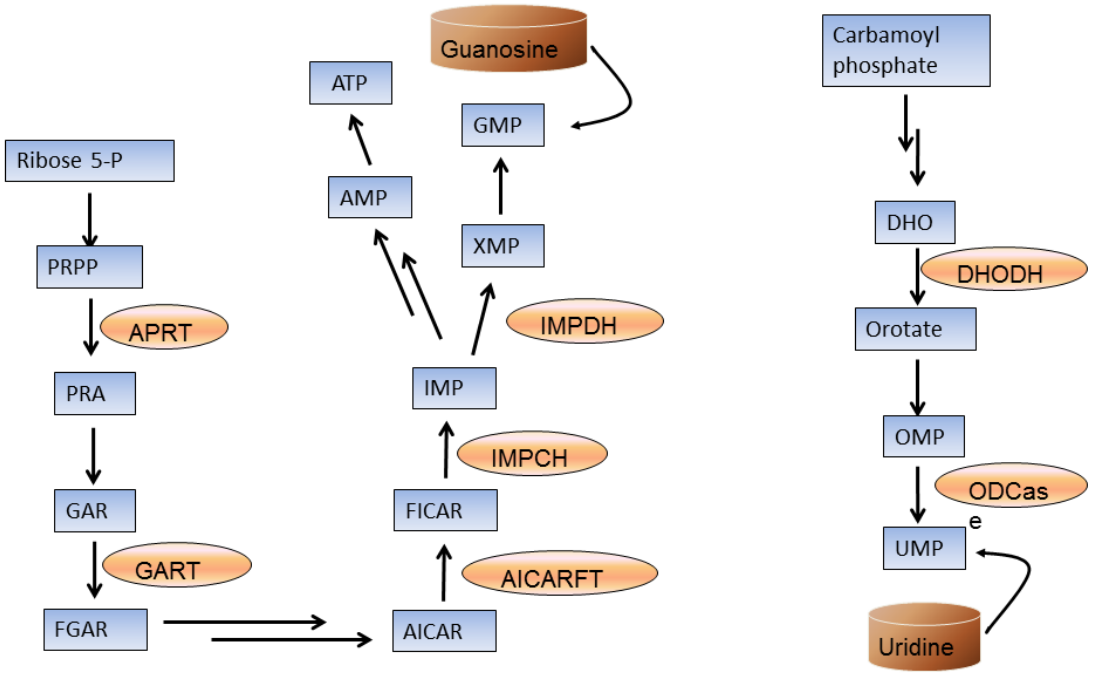

Figure 3. Schematic overview of *de novo* biosynthesis of purine and pyrimidine nucleotides. The blue boxes are intermediates in the *de novo* biosynthesis of purine and pyrimidine nucleotides. The oval boxes are catalyzed enzymes.

Viral replication is known to completely rely on the host to supply nucleosides essential for viral genesis. Inhibition of the *de novo* purine and pyridine biosynthesis pathway at any particular reaction provokes depletion of intermediates in the pathway, leading to an imbalance in nucleotide concentrations, in turn possibly affecting the life cycle of viral pathogens. The pathways of nucleotide biosynthesis thus become attractive targets for the clinical control of rapidly dividing cells such as cancers or infectious bacteria. Many antibiotics and anticancer drugs are inhibitors of purine or pyrimidine nucleotide biosynthesis. Thus, host enzymes participating in nucleoside biosynthesis are potential targets for antiviral therapeutic drug development. Many *de novo* nucleotides synthesis inhibitors have been reported to exert antiviral activity. Ribavirin and MPA, as IMPDH

inhibitors deplete the GTP pool and potently suppress dengue virus, yellow fever virus (YFV), hepatitis B, hepatitis C and hepatitis E virus [69-74]. Likewise, Brequinar and Leflunomide, targeting Dihydroorotate dehydrogenase (DHODH), an enzyme essential for *de novo* biosynthesis of urine nucleotides, has been shown to inhibit human polyomavirus type BK (BKV), YFV, and dengue virus. However, it remains questionable, with the possible exception of IMPDH, as to how the other catalytic enzymes involved in the two *de novo* nucleotides biosynthesis pathways regulate HEV replication. In **chapter 6**, I profiled these activities and chart the modulation of HEV replication by targeting catalytic enzymes involved in purine and pyrimidine synthesis, in an effort aimed at identifying promising targets for anti-HEV medication.

Interplay between mitochondria dynamics and virus infection

Mitochondria constitute a central element in cellular metabolism and are principal constituents of the molecular events involved in immune defense, apoptosis, autophagy and other signaling pathways [75]. Mitochondria are unique not only because they contain their genomic DNA, replicating independently from nuclei, but also because they are highly dynamic organelles that can be constantly undergo continuous cycles of homotypic fusion and fission, resulting in alteration of organelle shape, size, number, mitochondrial DNA stability and other signaling [76, 77] (Figure 4). These dynamic processes are sensitive to the alteration of cellular environmental or metabolic conditions, examples are oxygen stress, cancer, viral infection, a dynamism that appears essential to maintain cellular homeostasis [78-83]. Perturbations of the mitochondrial quality control machinery accordingly provoke substantial physiological consequences and are tightly related to pathogenesis of a number of genetic and neurological disorders, cardiac dysfunctions, cancer and metabolic diseases [84-88].

The two opposing events in mitochondrial dynamics, fusion and fission are ultimately coordinated by dynamin-related proteins. The main molecules governing mitochondrial fusion are optic atrophy 1 protein (OPA1), and Mitofusins (Mfn1 and Mfn2), at least in mammals. Mitochondrial fusion takes place on either outer or inner mitochondrial membranes to merge two individual mitochondria and quickly exchange and equilibrate matrix metabolites, intact mtDNA copies and mitochondrial membranes components. Outer

and inner mitochondrial fusion are orchestrated by Mitofusins and OPA1, respectively [89, 90]. In contrast to fusion-related proteins located in mitochondria, the key regulator protein Drp1 in mitochondrial fission localizes to the cytosol [91]. Other fission proteins, including mitochondrial fission factor (Mff), mid49 and Mid51, which mediates Drp1 recruitment to mitochondria are confined to the mitochondria.

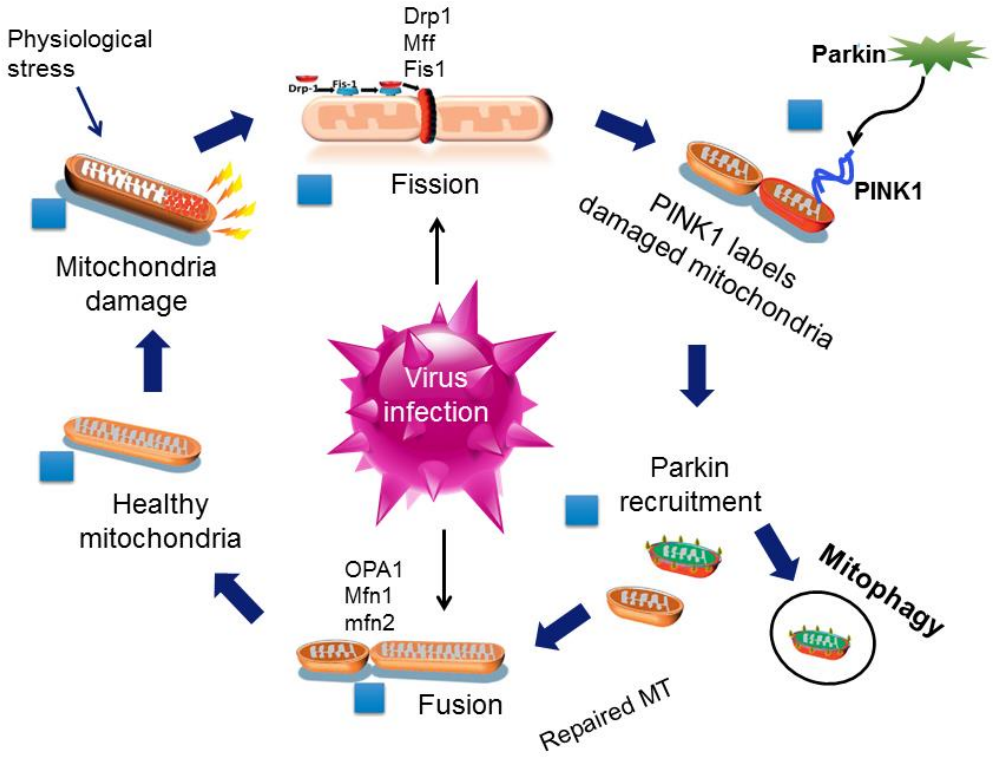

Figure 4. Overview of mitochondrial dynamics. Under normal physiological conditions, the mitochondria are usually tubular. Physiological or metabolic stress induces mitochondrial injury and causes mitochondrial impairment. The impaired part of the mitochondria is segregated by fission process. The impaired mitochondria are selectively flagged by the PINK1 that facilitates Parkin recruitment and mitophagy. All the repaired mitochondria are recruited to the functional mitochondrial network by fusion with other mitochondria (Adapted from Mohsin Khan, et al. Biochimica et Biophysica Acta, Review 2015.1 [83]).

In the past, the dysfunction of mitochondrial dynamics was initially reported to correlate with a myriad of neurodegenerative disease, in particular Alzheimer's, Huntington's and Parkinson's disease [92]. More recently, however, various infectious diseases, including HBV and HCV have been related to prominent mitochondrial injury as well [93-95]. The role of mitochondrial dynamics in viral infection is still highly controversial but the idea that exploring the role of mitochondrial dynamics in viral-host interaction might be promising and might provide promising targets for anti-viral therapy is gaining. Indeed, the

role of mitochondrial dynamics as a hub of innate immune signaling, cell apoptosis and mitophagy implicate that mitochondria represents a key factor in viral pathogenesis. Its relation, however, to HEV infection remains unexplored.

Virus infection modulates mitochondrial networks either through directly contact of the virus with mitochondria or by influencing mitochondrial morphology by altering physiological environment. Viruses may actively highjack such events to make host cells more permissive for their infection: activating mitophagy may e.g. impair cellular apoptosis and thus foster persistent viral infection while simultaneously suppress innate immune signaling. The common notion of the connection between mitochondrial dynamics and antiviral immunity is that mitochondrial fusion contributes to enhancement of MAVS signaling and IFN production, whereas mitochondrial fission dampens these innate immune response [96, 97]. Recent studies on HBV and HCV, reveal that both viruses can induce mitochondrial fission, leading to mitophagy and attenuating apoptosis of infected cells [95]. Moreover, the fission event also contributes to interference of virus triggered innate immune response to subvert mitochondrial associated antiviral signaling. Human cytomegalovirus was identified to stimulate mitochondrial fission and displayed anti-apoptosis properties [98]. For the *influenza virus*, on one hand, virus induces mitophagy, protecting host cells against virally triggered immunopathology [99]; on the other hand, influenza virus protein PB1-F2 downregulates innate immune response via directly targeting MAVS to affect IFN synthesis or via induce mitochondrial fragmentation [100]. One exception in this aspect is the *severe acute respiratory syndrome-coronavirus (SARS)*, which induces mitochondrial fusion, but impair MAVS signaling, indicating that other mechanisms may be operative [101]. Thus in this thesis, I investigate the mitochondrial dynamics following HEV infection and explore their significance for viral propagation.

Aim and outline of this thesis

The factors that determine HEV infection and progression are still ambiguous. In this thesis, I aim to develop potential anti-HEV therapy in cell culture models based on discovering host factors involved in HEV infection.

Immunosuppressive medication used in organ transplantation recipients is a risk factor for chronic HEV development. Despite sporadic clinical cases described HEV

progression in response to different common used immunosupprants, the lack of cohort and systemic analysis make it difficult to draw solid conclusion with respect to the association of different immunosuppressants and the outcome of HEV progression. Thus, in **chapter 2 and chapter 3**, I investigated how different immunosuppressants affect HEV infection in cell culture models. Combining with clinical case reports, **chapter 4** ties together the recent steps forward in manipulation of immunosuppressants in organ transplantation and emphasize the current clinical and experimental evidence regarding the key implications of immunosuppressants in chronic hepatitis E. The most frequently used option for the chronic HEV, ribavirin, is also a IMPDH inhibitor, but not an immunosuppressive drug. The antiviral activities of both IFN-α and Ribavirin against HEV replication in vitro were characterized in **chapter 5**. Of note, I found that ribavirin and MPA, both of which are IMPDH inhibitors, potently inhibited HEV infection. Considering its property of inhibiting purine nucleotide synthesis, I propose the inhibition of nucleotide synthesis might be an attractive strategy for anti-HEV therapy. Therefore, in **chapter 6**, I investigated the role of different enzymatic cascades of nucleotide biosynthesis in hepatitis E virus (HEV) replication. Considering that protein kinases are principal components of the machineries that orchestrate immune response against diverse pathogenic entities, knowledge of such pathways could prove exceedingly useful for the rational design of therapeutic avenues against HEV infection. **In chapter 7**, I comprehensively profile kinase-mediated cascades in cell–autonomous antiviral immunity on Huh7 based HEV replication cell model and we identified protein kinase C alpha (PKCα) as an important anti-HEV mediator. The interest of mitochondria in virology is gaining popularity as viral infection involves modulation of the dynamics of this organelle. In **chapter 8**, I describe the role of mitochondria dynamics in HEV infection, providing a novel avenue to explore the viral-host interaction. Finally in **chapter 9**, I summarize and discuss the results and describe future perspectives.

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CHAPTER 2

Calcineurin Inhibitors Stimulate and Mycophenolic Acid Inhibits Replication of Hepatitis E Virus

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Abstract

BACKGROUND & AIMS: Many recipients of organ transplants develop chronic hepatitis, due to infection with the hepatitis E virus (HEV). Although chronic HEV infection is generally associated with immunosuppressive therapies, little is known about how different immunosuppressants affect HEV infection.

METHODS: A subgenomic HEV replication model, in which expression of a luciferase reporter gene is measured, and a full-length infection model were used. We studied the effects of different immunosuppressants, including steroids, calcineurin inhibitors (tacrolimus [FK506] and cyclosporin A), and mycophenolic acid (MPA, an inhibitor of inosine monophosphate dehydrogenase) on HEV replication in human hepatoma cell line Huh7. Expression of cyclophilins A and B (the targets of cyclosporin A) were knocked down using small hairpin RNAs.

RESULTS: Steroids had no significant effect on HEV replication. Cyclosporin A promoted replication of HEV in the subgenomic and infectious models. Knockdown of cyclophilin A and B increased levels of HEV genomic RNA by 4.0 $\pm$ 0.6-fold and 7.2 $\pm$ 1.9-fold, respectively (n = 6; $P < 0.05$). A high dose of FK506 promoted infection of liver cells with HEV. In contrast, MPA inhibited HEV replication. Incubation of cells with guanosine blocked the antiviral activity of MPA, indicating that the antiviral effects of this drug involve nucleotide depletion. The combination of MPA and ribavirin had a greater ability to inhibit HEV replication than MPA or ribavirin alone.

CONCLUSIONS: Cyclophilins A and B inhibit replication of HEV; this might explain the ability of cyclosporin A to promote HEV infection. On the other hand, the immunosuppressant MPA inhibits HEV replication. These findings should be considered when physicians select immunosuppressive therapies for recipients of organ transplants who are infected with HEV.

Keywords: Cell Culture Model; Liver Disease; Immunity; Transplantation.

Introduction

Hepatitis E virus (HEV) is one of the most common causes of acute hepatitis worldwide. It is a single stranded positive-sense RNA virus that mainly infects the liver hepatocytes. Although only a single HEV serotype is recognized, at least 4 different genotypes of human HEV exist [1]. Genotypes 1 and 2 are found mainly in developing countries and are transmitted via contaminated water sources. In contrast, genotypes 3 and 4 are prevalent in industrialized countries and are zoonotic nature and spread mainly through eating undercooked pork or game products [2]. In general, HEV infection is a self-limiting disease and is associated with low mortality, but fulminant hepatitis and high mortality have been described, reaching 25% in cases of pregnant women infected with genotype 1 in developing countries [3]. In the Western world, the main clinical challenge is posed by HEV genotype 3 infection in patients receiving orthotopic organ transplantation [4]. More than 60% of organ recipients infected with HEV will develop chronic hepatitis with rapid progression to cirrhosis [5,6]. Which factors that determine outcomes in these patients remains obscure at best, hampering efforts to develop rational therapy and to address the increasing challenge of HEV infection in organ transplantation recipients.

Organ transplant patients take immunosuppressants for life to prevent graft rejection. The resulting immunosuppression, however, also affects host immunity against viral challenges, and the use of immunosuppressive drugs has been proposed to be a key factor for developing chronic hepatitis after HEV infection. Consequently, dose reduction of immunosuppression is often used as the first intervention strategy to achieve viral clearance in HEV-infected organ recipients [7]. Interestingly, however, clinical evidence suggests that different immunosuppressive regimens can differentially affect the infection course of HEV. The calcineurin inhibitor tacrolimus, but not cyclosporin A (CsA), has been found to be more frequently associated with persistent infection, and mycophenolate mofetil, the pre-drug form of mycophenolic acid (MPA) can help to clear the virus [8]. However, the current clinical studies are not able to conclusively address the impact of different immunosuppressants because of limited patient numbers and lack of mechanistic insight as to how differences in immunosuppressive medication might be linked with an altered clinical course of HEV infection.

The observation that different immunosuppressive medication seems to have specific effects on the outcomes of HEV infection suggests that such medication can have direct effects on viral replication, apart from influencing antiviral immunity. This consideration prompted us to test whether different immunosuppressive medication affects HEV replication in hepatocytes directly. The recent development of a genotype 3-based cell culture system [9,10] makes it possible to study such questions in a highly detailed fashion. We show that different commonly used immunosuppressants have very specific effects on viral replication and that especially calcineurin inhibitors strongly facilitate HEV replication, and MPA suppresses viral replication. These results will serve as an important reference about the choice of particular immunosuppressive medication for HEV infected orthotopic organ transplant recipients.

Materials and Methods

Immunosuppressants

CsA and tacrolimus (FK506) were purchased from Abcam (Cambridge, MA). Dexamethasone (Dex), prednisolone (Pred) and MPA were purchased from Sigma (St Louis, MO). All the reagents were dissolved in dimethyl sulfoxide, except MPA, which was dissolved in methanol. The effects of these immunosuppressants on host cell viability were determined by MTT assay (Supplementary Figure 1).

Cell culture

Human hepatoma cell line Huh7 and human embryonic kidney epithelial cell line 293T cells were cultured in Dulbecco's modified Eagle medium (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum, 100 IU/mL penicillin, and 100 IU/mL streptomycin.

Hepatitis E Virus Cell Culture Models

A plasmid construct containing the full-length HEV genome (Kernow-C1 p6 clone, GenBank Accession Number JQ679013) and a construct containing subgenomic HEV sequence coupled with a Gaussia luciferase reporter gene (p6-luc) were used to generate HEV genomic RNA by using the Ambion mMESSAGE mMACHINE *in vitro* RNA transcription

Kit (Life Technologies Corporation, Carlsbad, CA) [9,10]. Huh7 cells were electroporated with p6 full-length HEV RNA or p6-Luc subgenomic RNA to generate infectious or replication models, respectively [10].

Quantification of Hepatitis E Virus Infection

For the HEV replication model (p6-Luc), the activity of secreted gaussia luciferase in the cell culture medium was measured using BioLux Gaussia Luciferase Flex Assay Kit (New England Biolabs, Ipswich, MA), as quantification of viral replication. To further determine the specific effects on viral replication-related luciferase activity, Huh7 cells constitutively expressing the firefly luciferase reporter gene driven by the human PGK promoter were used as household luciferase activity for normalization [11]. For firefly luciferase, luciferin potassium salt (100 mM; Sigma) was added to cells and incubated for 30 minutes at 37 ℃. Both gaussia and firefly luciferase activity were quantified with a LumiStar Optima luminescence counter (BMG LabTech, Offenburg, Germany).

For the p6 infectious HEV model, SYBR Green–based quantitative real-time polymerase chain reaction (qRT-PCR) was used to quantify genomic RNA. The HEV primer sequences were 5'-ATTGGCCAGAAGTTGGTTTTCAC-3' (sense) and 5'-CCGTGGCTATAATTGTGGTCT-3' (antisense), and the primers of housekeeping gene glyceraldehyde-3-phosphate dehydrogenase were 5'-TGTCCCCACCCCCAATGTATC-30 (sense) and 50-CTCCGATGCCTGCTTCACTACCTT-3' (antisense).

Gene Knockdown by Lentiviral Vector Delivered Short-Hairpin RNA

Lentiviral vectors, targeting cyclophilin A (CypA), cyclophilin B (CypB) or green fluorescent protein, were produced in 293T cells as described previously.[12] After pilot study, the shRNA vectors exerting optimal gene knockdown were selected. The shRNA sequences were: CypA, 50-CCGGTGGTGACTTCACACGCCATAACTCGAGTTATGGCGTGTGAAGTCACCAT TTTTG-3', and CypB, 5'-CCGGGCCTTAGCTACAGGAGAGAAACTCGAGTTTCTCTCCTGTAGCTAAG GCTTTTTG-3'.

To generate stable gene knockdown cells, Huh7 cells were transduced with lentiviral vectors. Because the vectors also express a puromycin resistance gene, transduced cells were subsequently selected by adding 2.5 μg/mL puromycin (Sigma) in the cell culture

medium. For the infectious model, HEV particles were incubated with knockdown and control Huh7 cells. For the subgenomic model, p6-Luc cells were directly transduced with lentiviral shRNA vectors and selected by adding 2.5 µg/mL puromycin.

Western Blot

For Western blot, commercial antibodies against CypA and CypB (rabbit polyclonal; Abcam) were used. Proteins in cell lysates were heated 5 minutes at 95℃, followed by loading onto a 15% sodium dodecyl sulfate polyacrylamide gel and separating by electrophoresis. After 90 minutes running in 115-V voltage, proteins were electrophoretically transferred onto a polyvinylidene difluoride membrane (Invitrogen) for 1.5 hours with an electric current of 250 mA. Subsequently, the membrane was blocked with 2.5 mL blocking buffer and 2.5 mL phosphate-buffered saline containing 0.05% Tween 20. It was followed by incubation with rabbit anti-CypA (1:5000) or anti-CypB (1:7500) antibody overnight at -4℃. Membrane was washed 3 times followed by incubation for 1.5 hours with an anti-rabbit peroxidase–conjugated secondary antibody (1:5000). After washing 3 times, protein bands were detected with Odyssey 3.0 Infrared Imaging System.

Statistical Analysis

Statistical analysis was performed using the nonpaired, nonparametric test (Mann-Whitney test; GraphPad Prism software, GraphPad Software Inc., La Jolla, CA). P values <0.05 were considered statistically significant.

Results

Glucocorticosteroids Did Not Affect Hepatitis E Virus Replication

Pred and its close analogue Dex remain important drugs in the clinical management of patients receiving orthotopic organ transplantation [13]. To study the possible effects of these drugs on HEV replication, we used a model in which cells were transfected with a subgenomic construct of HEV coding sequence in which the 5’ portion of ORF2 was replaced with the in-frame secreted form of luciferase derived from the marine copepod Gaussia princeps. Accumulation of luciferase serves as reporter for HEV RNA synthesis (p6-luc), and the loss of the capsid protein precludes the formation of novel viral particles. In parallel,

Huh7 cells constitutively expressing a nonsecreted firefly luciferase were used for normalization of nonspecific effects on luciferase signals. However, as shown in Figure 1 neither Pred nor Dex significantly affected HEV replication. We conclude that steroids have no direct effects on HEV replication.

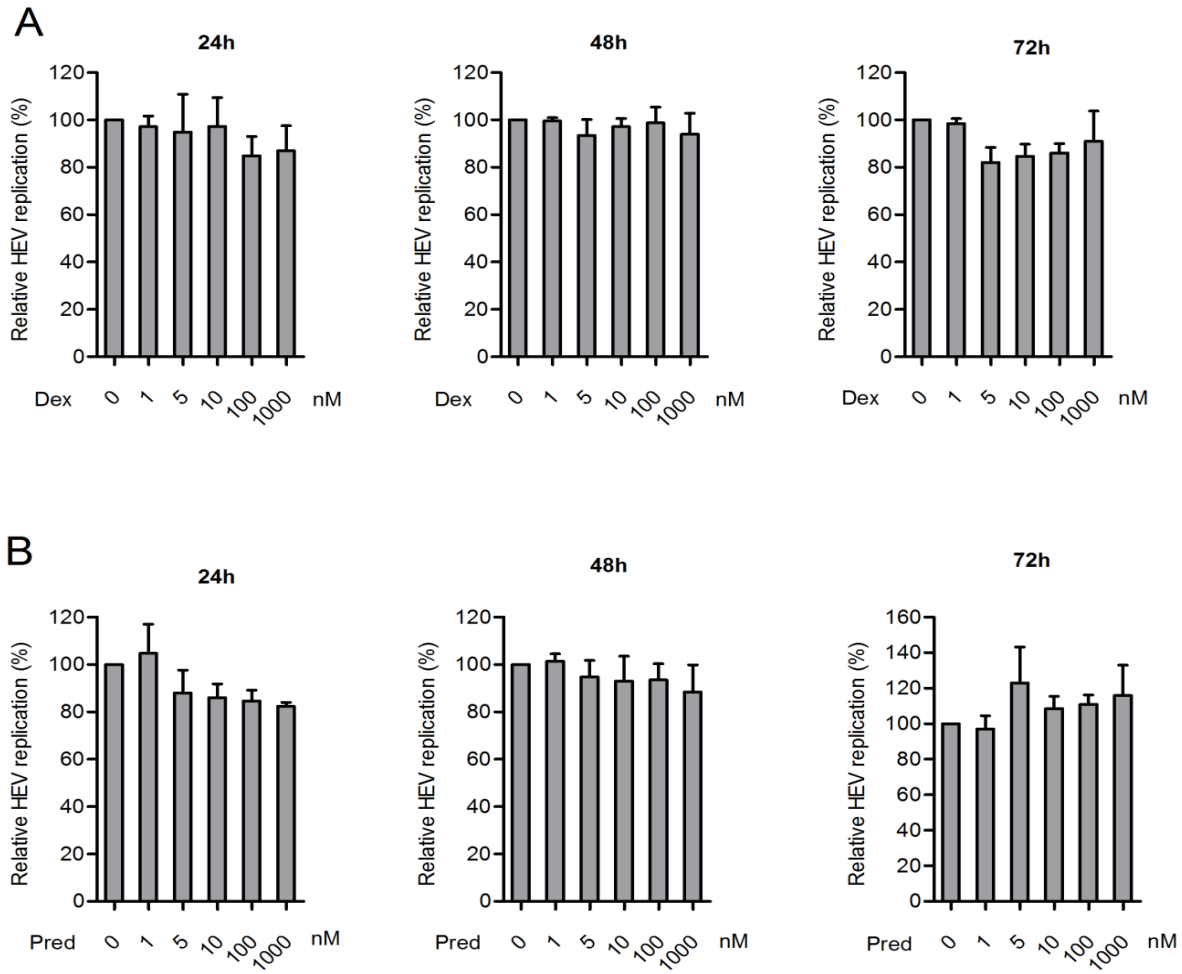

Figure 1. The effects of steroids on HEV replication in subgenomic cell culture model. Huh7 cell based subgenomic HEV replicon containing the luciferase reporter gene was treated for 24, 48, and 72 hours with a dose-range of Dex and Pred. (A) Dex and (B) Pred did not significantly affect luciferase activity. Data presented as mean ± SD of 3 independent experiments.

Cyclosporin A Dose-Dependently Enhanced Hepatitis E Virus Replication

CsA, a calcineurin inhibitor, is an important drug for prevention of graft rejection. To examine the effects of CsA on HEV replication, we tested the effects of 0.1, 0.5, and 5 µg/mL CsA on viral replication using the subgenomic p6-Luc model as a read-out. It appeared that CsA dose dependently increased HEV replication-related luciferase activity (Figure 2A). Consistently, CsA also dose dependently increased HEV infection in the full-length (p6)

infectious model (Figure 2B). Forty-eight hours of treatment with CsA (5 µg/mL) resulted in a mean SD of 2.67 ± 0.7-fold (n = 5; P < 0.01) increase of HEV genomic RNA level (determined by qRT-PCR), compared with the control (Figure 2B). CsA directly promotes viral replication in a hepatocyte-like cells and experimentation was initiated to establish the molecular basis of this effect.

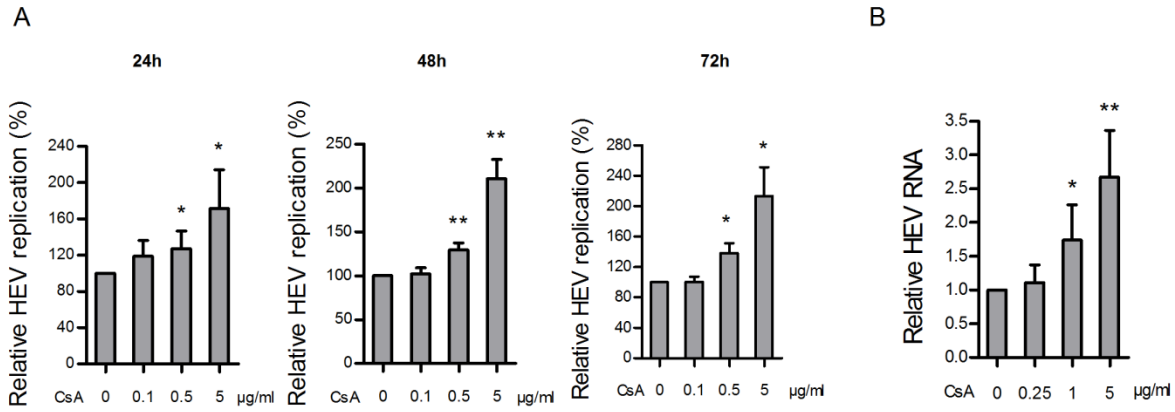

Figure 2. CsA promoted HEV infection. (A) The subgenomic HEV replicon containing the luciferase reporter gene was treated for 24 hours (n = 5), 48 hours (n = 7), and 72 hours (n = 7) with different doses of CsA. Treatment with CsA (0.5 or 5 µg/mL) significantly increased HEV luciferase activity. (B) The Huh7 cells-based infectious HEV model was treated with CsA for 48 hours. CsA significantly increased HEV RNA at 0.5 and 5 µg/mL concentrations (n = 5). Data presented as mean ± SD of multiple experiments. * P < 0.05; ** P <0 .01.

Silencing the Cellular Targets of Cyclosporin A, Cyclophilin A and B, Enhanced Hepatitis E Virus Replication

CsA acts through binding and inhibition of the CypA/B complex. The effects of CsA on HEV replication could be potentially mediated through cyclophilins. CypA [14] and CypB [15] have been implicated in the anti–hepatitis C virus (HCV) mechanism of CsA. Therefore, lentiviral-mediated RNA interference was used for knockdown of these 2 genes, as to allow investigation of their potential function in the effects of CsA on HEV replication. To this end, Huh7 cells were transduced with integrating lentiviral vectors expressing both shRNA and puromycin. Cells stably transduced with the vector were selected and expanded by adding puromycin to the relevant cell cultures. The shRNA clones with most potent efficacy of CypA and CypB knockdown were selected for follow-up experimentation (Figure 3A). Cells stably integrated with shRNA targeting GFP (as control), CypA or CypB were inoculated with infectious HEV viruses (p6). The level of infection was quantified by qRT-PCR of genomic viral RNA in the cells 3 days post inoculation. As shown in Figure 3B, knockdown of CypA

resulted in a 4.0 ± 0.6-fold (n = 6; P < 0.01) increase of HEV RNA; and knockdown of CypB has resulted in a 7.4 ± 1.9-fold (n = 6; P < 0.05) increase of viral genomic RNA. Consistently, silencing of CypA and CypB in HEV subgenomic model significantly increased viral replication-related luciferase activity by a mean ± SEM of 350.4% ± 11.7% (n = 12; P < 0.001) and 406% ± 14.5% (n = 12; P < 0.001), respectively (Figure 3C). The most straightforward explanation of these results is that CsA through cyclophilin binding and inhibition facilitates HEV infection (Figure 2).

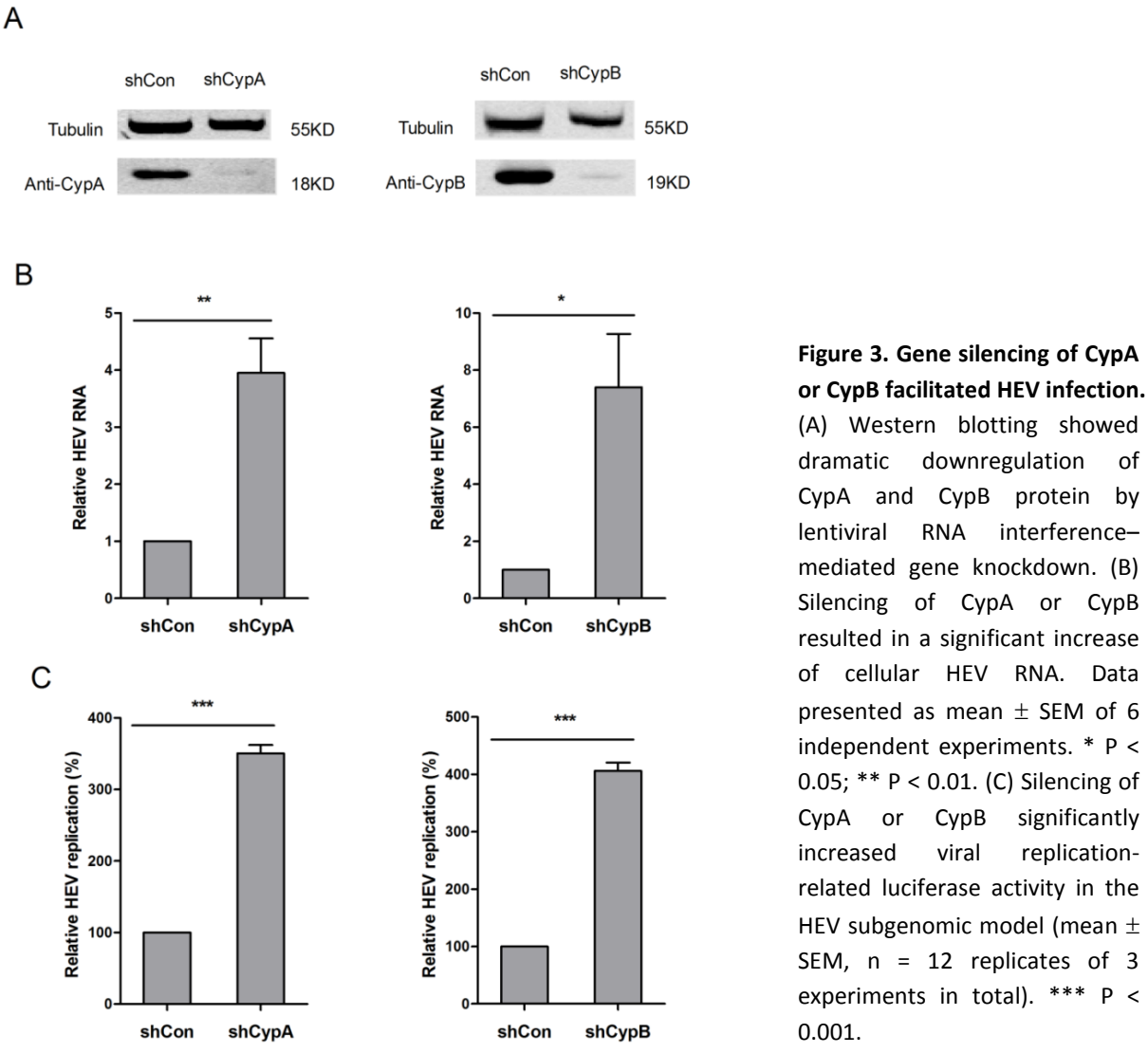

Figure 3. Gene silencing of CypA or CypB facilitated HEV infection. (A) Western blotting showed dramatic downregulation of CypA and CypB protein by lentiviral RNA interference–mediated gene knockdown. (B) Silencing of CypA or CypB resulted in a significant increase of cellular HEV RNA. Data presented as mean ± SEM of 6 independent experiments. * P < 0.05; ** P < 0.01. (C) Silencing of CypA or CypB significantly increased viral replication-related luciferase activity in the HEV subgenomic model (mean ± SEM, n = 12 replicates of 3 experiments in total). *** P < 0.001.

High Dose of FK506 Promoted Hepatitis E Virus Replication

FK506 is another type of calcineurin inhibitor that binds to FK binding proteins. To determine the effects of FK506 on HEV replication, p6-Luc cells were treated with FK506 at concentrations of 0.1, 0.5, and 5 µg/mL. As shown in Figure 4A, only high does (5 µg/mL) of FK506 significantly increased HEV replication, seen at 24, 48, and 72 hours post-treatment. This was also further confirmed in the p6 infectious model that HEV genomic RNA was increased by a mean SD of 35% ± 9.6% (n = 4; P < 0.01) by treatment with 5 µg/mL FK506 for 48 hours (Figure 4B).

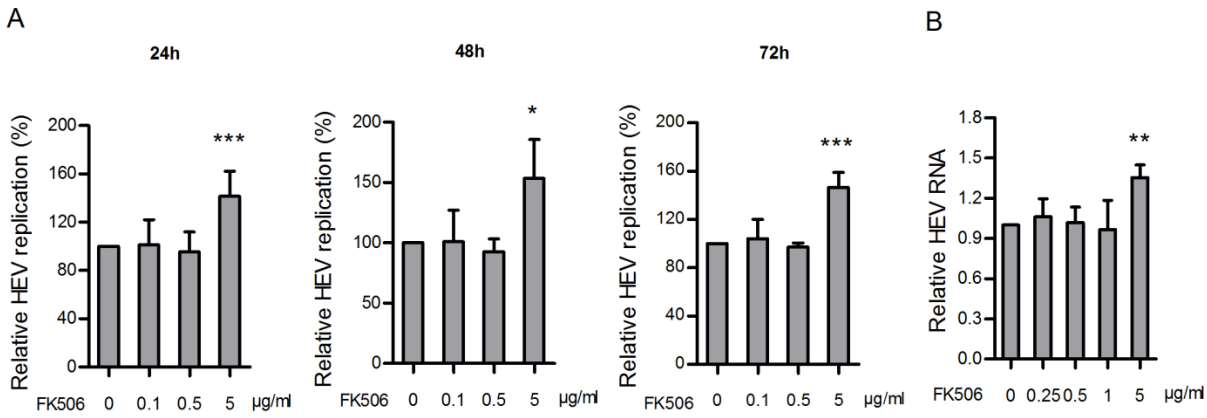

Figure 4. High dose of FK506 enhanced HEV infection. (A) Treatment with 5 µg/mL (but not 0.5 and 1 µg/mL) resulted in significant increase of luciferase activity in the HEV subgenomic model (mean ± SD; n = 5-8) and (B) significant increase of HEV RNA in the infectious model (mean ± SD, n = 4). * P < 0.05; ** P < 0.01; *** P < 0.001.

Because the immunosuppressive mechanisms of calcineurin inhibitors are mediated via the Ca^{2+} -NFAT signal transduction, we tested the effects of N,N-dimethyl-Derythro-sphingosine, a compound that can efficiently increase cellular Ca^{2+} levels [16], on HEV infection. As shown in Supplementary Figure 2A, N,N-dimethyl-D-erythro-sphingosine (1- 4 µg/mL) triggered clear induction of Ca^{2+} levels in Huh7 cells visualized with a fluorescent dye, Fluo-4/AM. However, no clear effects were observed on HEV infection in either the subgenomic (Supplementary Figure 2B) or the infectious (Supplementary Figure 2B) model. Therefore, the proviral effects of calcineurin inhibitors on HEV infection appear to be independent of Ca^{2+} levels.

Mycophenolic Acid Inhibited Hepatitis E Virus Replication by Depletion of Cellular Nucleotide Pool

MPA, an inhibitor of inosine monophosphate dehydrogenase (IMPDH) (the biosynthesis of guanine), is an immunosuppressive drug often used in organ transplantation, but also has a broad antiviral activity against a spectrum of viruses [17]. We investigated whether MPA could also be able to inhibit HEV infection. Treatment with MPA (0.1-10 μg/mL) has resulted in a significant reduction of HEV replication-related luciferase activity in the subgenomic replicon. For example, with 10 μg/mL MPA treatment, the luciferase activity were 42.8% ± 2.3% (mean ± SEM) (n = 9; P < 0.001), 32.8% ± 5.3% (n = 10; P < 0.001), and 39.5% ± 4.6% (n = 12; P < 0.001) of the control group at days 1, 2, and 3, respectively (Figure 5A). Consistently, MPA also dose-dependently inhibited cellular viral RNA in the infectious HEV model. Forty-eight hours of treatment with MPA (10 μg/mL) resulted in 65% ± 9% (n = 5; P < 0.01) inhibition of HEV genomic RNA level (determined by qRT-PCR) compared with the control (Figure 5B)

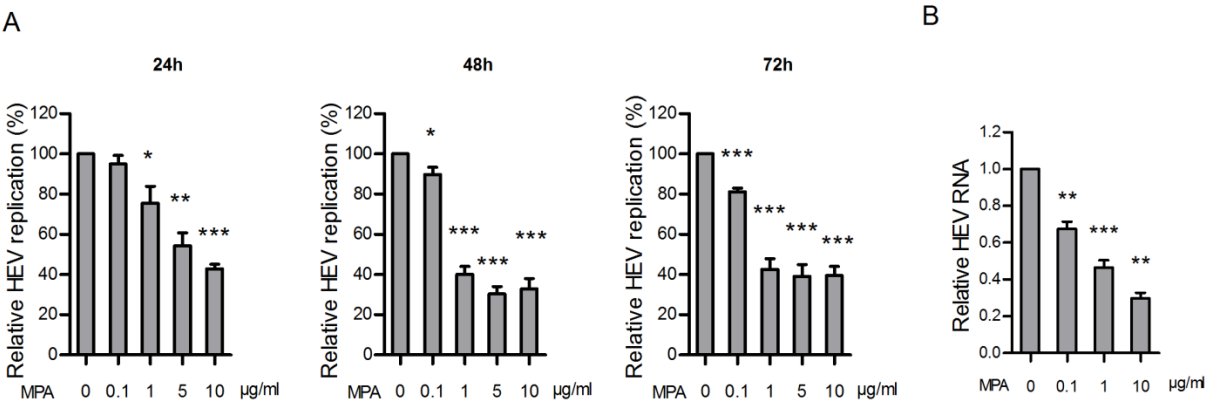

Figure 5. Potent anti-HEV activity of MPA. (A) Treatment of MPA for 24, 48, or 72 hours has resulted in significant reduction of HEV luciferase activity in the subgenomic model (mean ± SEM, n = 9-12). (B) In the infectious model, treatment with 0.1, 1, and 10 μg/mL of MPA for 48 hours has significantly inhibited HEV RNA by 32%, 57%, and 65%, respectively (mean ± SEM, n = 5). * P < 0.05; ** P < 0.01; *** P < 0.001.

To further investigate whether the effects of MPA are via depletion of cellular nucleotides, additional guanosine was added to the MPA treatment. As shown in Figure 6, supplement of exogenous guanosine completely abrogated the antiviral activity of MPA in both subgenomic and infectious HEV models, suggesting that the action of MPA is exclusively via nucleotide depletion. Immunosuppressive drugs have highly diverse effects

on HEV replication, calcineurin inhibitors stimulating viral replication, but MPA exerting direct inhibition of HEV replication.

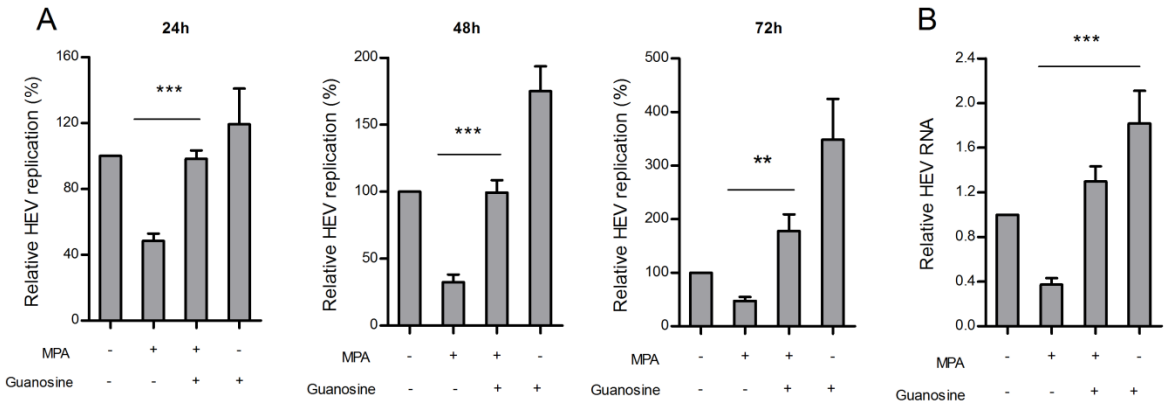

Figure 6. Supplement of exogenous guanosine completely abrogated the anti-HEV effects of MPA. (A) In the subgenomic HEV replicon, the antiviral effects by treatment of MPA at concentration of 10 μg/mL for 24 hours, 48 hours, and 72 hours were abrogated by adding exogenous guanosine (100 μg/mL) (mean ± SEM, n = 7-10). (B) Similarly, the antiviral effects by treatment of MPA at concentration of 10 μg/mL for 48 hours was also abrogated by adding 100 μg/mL exogenous guanosine in the infectious model (mean ± SEM, n = 8). ** $P < 0.01$; *** $P < 0.001$.

Combination of Mycophenolic Acid With Ribavirin Extended Their Antiviral Activity

Because the use of ribavirin monotherapy as off-label drug is gaining favor for treating hepatitis E [18], we also investigated the antiviral effects of combining MPA with Ribavirin. As shown in Figure 7, a serial of combination groups demonstrated a general beneficial effect and no negative drug–drug interference was observed. For instance, combining 1 μg/mL MPA with 25 μm ribavirin resulted in a mean SEM of 76% ± 1% inhibition of HEV luciferase, and MPA alone resulted in 60% ± 2% and ribavirin alone resulted in 17% ± 3% inhibition (n = 16; $P < 0.001$) after 72 hours treatment (Figure 7A). Therefore, a combination of ribavirin with MPA appears compatible against HEV infection and constitutes an attractive clinical option for preventing rejection in HEV-infected patients.

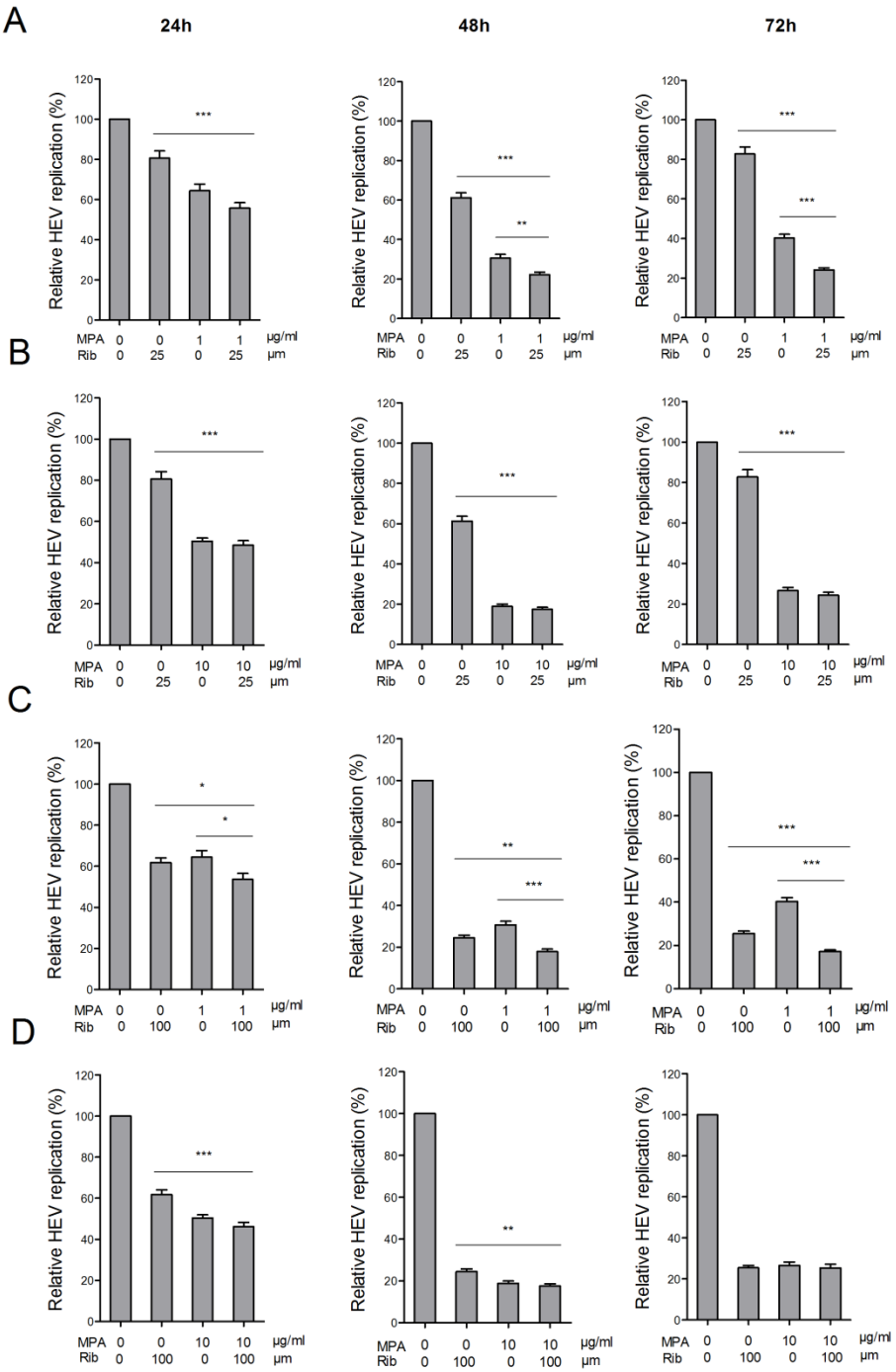

Figure 7. Combination of MPA with ribavirin extended their antiviral activity. Treatment with ribavirin alone has showed significant anti-HEV effects (mean ± SEM, n = 16 replicates in total) and a combination of MPA with ribavirin demonstrated an additional antiviral potency in particular combination groups; MPA doses: 1 µg/mL; 10 µg/mL; ribavirin doses: 25 µm; 100 µm. (A) 1 µg/ml MPA combined with 25 µm ribavirin. (B) 10 µg/ml MPA combined with 25 µm ribavirin. (C) 1 µg/mL MPA combined with 100 µm ribavirin. (D) 10 µg/mL MPA combined with 100 µm ribavirin. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

Discussion

Immunosuppressive medication has been proposed to be a key factor for developing chronic hepatitis E in organ transplantation recipients [4] and is often solely attributed to diminished antiviral immunity. Clinical evidence, however, suggests that different immunosuppressive regimens can differentially affect the infection course of HEV [6,8]. By testing different immunosuppressants in 2 HEV replication models, we have consistently demonstrated that steroids (Pred and Dex) did not affect viral replication, calcineurin inhibitors (CsA and FK506) promoted HEV infection, and MPA suppressed viral infection *in vitro*. The concentrations of these immunosuppressants used in this study are in general covering the achievable blood concentrations in patients [19-21]. Of note, animal studies have indicated that certain immunosuppressants even accelerate in the liver and drug levels in hepatocytes will exceed those observed in serum [22]. Therefore, we propose that the results of this *in vitro* study will be a valuable reference regarding the choice of the particular immunosuppressant for orthotopic organ transplantation patients who are infected with HEV.

Steroids have been used since the early years of organ transplantation. Pred and its close analogue Dex are potent suppressors of the immune system, as they modulate cellular and inflammatory responses via stimulation or inhibition of gene transcription [23]. In the setting of liver transplantation for HCV patients, evidence suggested that steroid boluses used to treat acute rejection are associated with an increase in viral load and the severity of HCV recurrence [24,25]. Using subgenomic cell culture model of HCV replicon, a study demonstrated that both Pred and Dex have no stimulatory effect on viral RNA levels, but rather have minor inhibitory effects [13]. As to infectious HCV model, however, Pred was reported to promote HCV infection by enhancing virus entry, including up-regulation of 2 essential HCV entry factors: occluding and scavenger receptor class B type I [26]. In both subgenomic and infectious models of HEV, we did not observe clear effect on HEV infection by either Pred or Dex. Although limited studies have reported the impact of steroids in HEV patients, one case report has documented a good clinical and biochemical response to steroid therapy in a patient with acute hepatitis E with autoimmune hepatitis, who maintained health with low dose of steroids [27].

The first *in vitro* evidence that CsA but not FK506 can inhibit HCV replication [28] sparked the clinical debate on the possible differential effects of these 2 drugs on HCV recurrence after liver transplantation [29]. Several follow-up studies have demonstrated that the targets of CsA, CypA, and CypB are host factors supporting HCV infection [14,15] .CsA exerts anti-HCV effects by inhibition of these cellular factors [30]. Interestingly, we observed a proviral effect of CsA in HEV cell culture models. Using RNA interference gene silencing approach, we further demonstrated that knockdown of either CypA or CypB enhanced HEV infection, suggesting that both factors could restrict HEV infection. This convincingly explained why CsA could facilitate HEV infection. Although a number of reports have demonstrated a supportive role of CypA in infections of HIV,[31] HCV,[14] or HBV,[32] recent studies also reported that CypA possesses a repressive effect on the replication of some viruses, including influenza A virus [33] and rotavirus [34], similar to what we have observed for HEV. Because the mechanistic insight is still largely missing for the antiviral action of cyclophilins, it deserves additional investigation. In addition, we also observed a proviral effects of FK506, but only at high dose. To our knowledge, there is no evidence of FK506 affecting HCV infection in cell culture [28,35]. In fact, compared with CsA, dose reduction of FK506 was assumed to be associated more with clearance of HEV in cases of renal transplantation with acute infection [36]. In a large retrospective study (although only 85 patients were included), the use of FK506 was the main predictive factor for chronic hepatitis E in organ recipients [6]. Our *in vitro* results have indicated that both FK506 and CsA can promote HEV infection. However, these data do not necessarily contradict to the clinical observation, because the number of patients currently investigated in the clinic is rather too small to draw solid conclusion. In addition, besides the direct effects we observed in cell culture, drugs can also have indirect influence on the infection.

The antiviral effects of MPA/mycophenolate mofetil have been demonstrated against a broad spectrum of viruses, including Dengue virus, West Nile, yellow fever virus, Chikungunya virus, HBV, and HCV [36–39]. This is consistent with our finding that MPA also potently inhibited HEV replication. For several viruses, MPA exerts antiviral effects by targeting IMPDH to deplete cellular nucleotide pools [36]. In the case of HCV, the IMPDH-dependent pathway only partially contributed to its antiviral activity [11]. In contrast, supplementation of exogenous guanosine completely abrogated the anti-HEV activity of

MPA, suggesting a crucial role of IMPDH inhibition leading to depletion of cellular nucleotides. Interestingly, clearance of HEV after heart transplantation was found to be more frequent in patients with immunosuppressive medication containing MMF [8], although this might be biased by a reduced dose of CsA or FK506 in these cases.

Despite a clear benefit to manipulating immunosuppressive regimens, a substantial proportion of patients are still not able to clear the virus and rapidly progresses toward chronic hepatitis [6]. Although no proven medication is available, the use of ribavirin monotherapy as off-label drug is gaining acceptance for treating hepatitis E [18]. An intriguing question is whether immunosuppressants can interfere with or promote the anti-HEV efficacy of ribavirin. In this study, we have finally demonstrated a beneficial effect of combining ribavirin with MPA (Figure 7). This does provide a proof of concept that it is important to choose the right immunosuppressive medication while under antiviral therapy of HEV in organ transplant recipients.

different immunosuppressants on HEV infection in cell culture. Steroids did not affect genotype 3 HEV replication *in vitro*, but a high dose of FK506 promoted HEV infection. CsA dose-dependently facilitated HEV infection by targeting cellular factors CypA and CypB. In contract, MPA potently suppressed HEV infection by depletion of cellular nucleotide pools. In addition, a clear beneficial effect was observed when MPA combined with another antiviral regimen ribavirin. Although experimental research alone will not be able to clarify these complicated but important clinical issues, the knowledge gained from this study is certainly a valuable reference for the management of immunosuppression in organ transplant recipients infected with HEV. Hopefully, it will also promote the initiation of randomized controlled clinical studies to address these issues in the near future.

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Supplementary Materials

Methods:

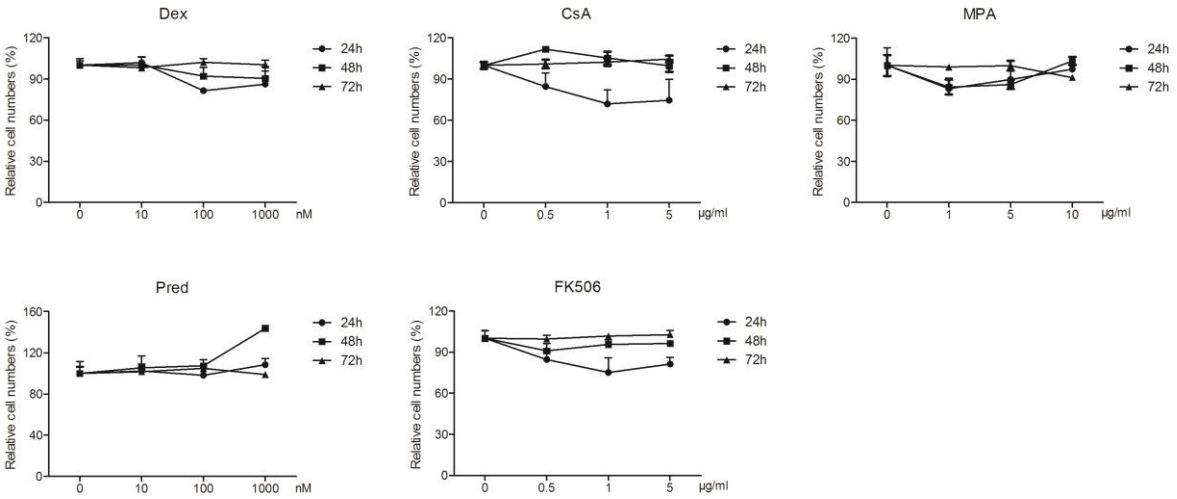
MTT assay

Huh7 cells were plated in 96-well plates and treated with immunosuppressants. At the indicated times, the number of metabolically active cells was quantified by the 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT, 0.5 mg/mL) assay.

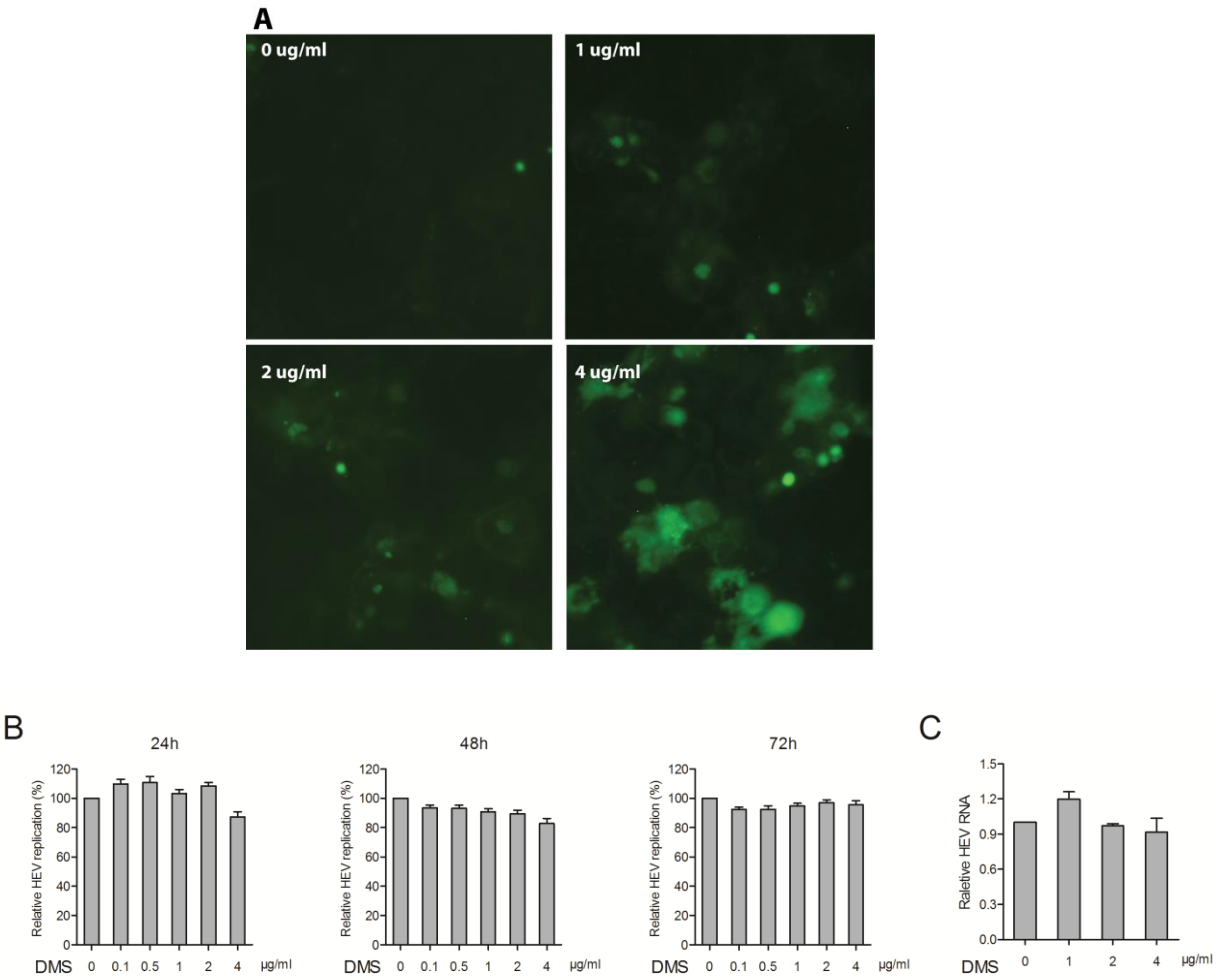
Measurement of [Ca^{2+}] concentration

The intracellular [Ca2+] concentration was measured using the fluorescent dye, Fluo-4/AM (Dojindo Laboratories, Kumamoto, Japan). The Huh7 cells were treated with serial dilutions of DMS for 48 h, then resuspended in PBS containing 1% bovine serum and incubated for 30 min with 5 μM Fluo-4/AM in the dark. After being washed with PBS, the Fluo-4/AM-labeled cells were observed under an inverted fluorescence microscope.

Figures and Legends:



Supplementary Figure 1. The effects of immunosuppressants on viability of Huh-7 cells. Cells were treated with immunosupprants at different concentrations for 24, 48 and 72 h. Cell viability was assayed by the MTT test. Shown is Mean ± SD from at least 3 independent experiments.



Supplement Figure 2. Induction of cellular Ca2+ concentration by N,N-Dimethyl-D-erythro-sphingosine (DMS) did not affect HEV infection. (A) DMS (1-4 µg/ml) triggered clear induction of Ca2+ levels in Huh7 cells visualized with a fluorescent dye, Fluo-4/AM. (B) HEV replication in the subgenomic model was not affected by DMS treatment for 24, 48 and 72h, respectively. (C) HEV infection was also not affected by DMS treatment for 48 hrs in the infectious model quantified by qRT-PCR. Shown is Mean ± SD from 3 independent experiments.

CHAPTER 3

Rapamycin and Everolimus Facilitate Hepatitis E Virus Replication: Revealing a Basal Defense Mechanism of PI3K-PKB-mTOR Pathway

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Abstract

BACKGROUND & AIMS: Humans are frequently exposed to hepatitis E virus (HEV). Nevertheless, the disease mainly affects pregnant women and immunocompromised individuals. Organ recipients receiving immunosuppressants, such as rapalogs, to prevent rejection have a high risk for developing chronic hepatitis following HEV infection. Rapalogs constitute potent inhibitors of mTOR including rapamycin and everolimus. As a master kinase, the mechanism-of-action of mTOR is not only associated with the immunosuppressive capacity of rapalogs but is also tightly regulated during pregnancy because of increased nutritional demands.

METHODS: We thus investigated the role of mTOR in HEV infection by using two state-of-the art cell culture models: a subgenomic HEV containing luciferase reporter and a full-length HEV infectious cell culture system.

RESULTS: In both subgenomic and full-length HEV models, HEV infection was aggressively escalated by treatment of rapamycin or everolimus. Inhibition of mTOR was confirmed by Western blot showing the inhibition of its downstream target, S6 phosphorylation. Consistently, stable silencing of mTOR by lentiviral RNAi resulted in a significant increase in intracellular HEV RNA, suggesting an antiviral function of mTOR in HEV infection. By targeting a series of other up- and downstream elements of mTOR signaling, we further revealed an effective basal defense mechanism of the PI3K-PKB-mTOR pathway against HEV, which is through the phosphorylated eIF4E-binding protein 1 (4E-BP1), however independent of autophagy formation.

CONCLUSIONS: The discovery that PI3K-PKB-mTOR pathway limits HEV infection through 4E-BP1 and acts as a gate-keeper in human HEV target cells bears significant implications in managing immunosuppression in HEV-infected organ transplantation recipients.

Keywords: Hepatitis E virus; Rapamycin; Everolimus; PI3K-PKB-mTOR pathway.

Introduction

Although hepatitis E virus (HEV) infection is underdiagnosed, it is clear that the virus represents one of the most abundant infectious challenges to humans [1]. In Western countries, HEV infection of healthy individuals almost exclusively remains subclinical and otherwise causes an acute and self-limiting infection in immunocompetent individuals with low mortality rates [2]. In contrast, patients with HEV infection in immunocompromised individuals that include organ transplantation recipients [3], HIV patients [4] and cancer patients receiving chemotherapy [5] have a substantially high risk of developing chronic hepatitis. The use of immunosuppressants, such as rapalogs, in organ transplant recipients to prevent rejection is associated with substantial pathology and in particular an increased risk of developing chronic hepatitis with substantial graft loss and mortality rates [6].

However, in undernourished populations in the developing world, fulminant hepatitis and high mortality are described, reaching 25% in the case of pregnant women [7]. In the current (2012–2013) hepatitis E outbreak among refugees in South Sudan, a total of 5080 acute jaundice syndrome cases had been reported from all four Maban County refugee camps, as of January 27, 2013. An acute jaundice syndrome case-fatality rate of 10.4% was observed among pregnant women across all camps [8]. Humans appear to have powerful HEV combating mechanisms, but these apparently require a good nutritional and host defence status for optimal functionality [9]. The nature of these mechanisms has not been characterised, due to the lack of robust HEV cell culture models. The advent of new technology that mimics the HEV infectious process *in vivo*, in particular the development of *in vitro* adapted infectious clones and subgenomic HEV reporters, has led to hopes that the mechanisms that control HEV infection in normal physiology can now be identified [10,11].

Rapalogs comprise, amongst others rapamycin (RAPA, rapamune, sirolimus; originally isolated from *Streptomyces hygroscopicus*) and everolimus (the 40-O-[2-hydroxyethyl] derivative of rapamycin). This immunosuppressive medication is gaining increasing popularity in the transplantation context, mainly because of its low nephrotoxicity [12]. Their molecular mode of action is well characterised and involves inhibition of the mammalian target of rapamycin (mTOR) pathway. mTOR is a central element within the phosphatidylinositol-3 kinase (PI3K)-protein kinase B (PKB)-mTOR signaling [13] and integrates

nutritional information and receptor tyrosine kinase signaling to control cellular growth via a variety of cellular effectors, including activation of p70 S6 kinase and subsequent protein synthesis as well as inhibition of autophagy. Activation of PI3K-PKB-mTOR signaling following viral infection of liver cells has been reported and linked to both viral supportive functions (e.g., prevention of apoptosis in hepatitis C-infected cells) [14], but also to the induction of the production of antiviral interferons [15]. Thus, generally speaking the role of this signaling cascade in combating viral infection of the liver remains unclear, prompting further research.

Given the important and increasing role of rapalog implications in clinical practice and the lack of insight into the mechanisms employed by the body to constrain HEV infection, we investigated the role of the PI3K-PKB-mTOR signaling cascade in HEV infection using state-of-the-art cell culture models. These results show that mTOR inhibition drastically promotes HEV replication in an autophagy-independent fashion but through phosphorylated 4E-BP1 in infected hepatocytes.

Materials and Methods

Reagents

Stocks of rapamycin (Merck, Schiphol-Rijk, The Netherlands) and everolimus (Sigma-Aldrich, St Louis, MO) were dissolved in dimethyl sulfoxide (DMSO) (Sigma-Aldrich, St Louis, MO) with a final concentration of 2 mM. Stocks of LY294022, an inhibitor of PI3K-PKB (Sigma-Aldrich), BEZ235, a dual inhibitor of PI3K-PKB and mTOR (Selleck Chemicals), FG-4592, an inhibitor of HIF-1a (Selleck Chemicals) and PF-478671, an inhibitor of p70 S6 kinase (Selleck Chemicals) were dissolved in DMSO. All agents were stored in 15 ll aliquots and frozen at -4℃. Antibodies including LC3-I/II (Cell Signalling Technology, Netherlands), S6, phospho- S6, p70 S6 kinase, phospho-PKB, 4E-BP1 and β-actin (Santa Cruz Biotech, Santa Cruz, CA); anti-rabbit or anti-mouse IRDye-conjugated secondary antibodies (Stressgen, Glandford Ave, Victoria, BC, Canada) were used for Western blot. Lentiviral particles of GFP-LC3-II (Millipore, Billerica, MA, USA), expressing GFPLC3 fusion protein, were used for visualisation of autophagy formation. Other reagents including EBSS medium (Lonza), E-64-d (Santa Cruz Biotech, Santa Cruz, CA), pepstatin A (Santa Cruz Biotech, Santa Cruz, CA) and chloroquine (Sigma–Aldrich) were also used.

HEV cell culture models

HEV genomic RNA was generated from a plasmid construct containing the full-length HEV genome (Kernow-C1 p6 clone, GenBank Accession Number JQ679013) or a construct containing subgenomic HEV sequence coupled with a Gaussia luciferase reporter gene (p6-luc), using the Ambion mMESSAGE mMACHINE *in vitro* RNA transcription Kit (Life Technologies Corporation) [10,11]. The human hepatoma Huh7 cells were collected and centrifuged for 5 min, 1500 rpm, -4 ℃. Supernatant was removed and washed with 4 ml Optimem by centrifuging for 5 min, 1500 rpm, -4 ℃. The cell pellet was re-suspended in 100 µL Optimem and mixed with p6 full-length HEV RNA or p6-Luc subgenomic RNA. Electroporation was performed with the Bio-Rad's electroporation systems using the protocol of a designed program (240 volt, pulse length 0.5, number 1 and cuvette 4 mm) [10]. The supernatant of cultured p6 full-length HEV RNA electroporated cells was collected and used for secondary infection.

Cell culture

Naïve or vector transduced HuH7 cells (human hepatoma cell line) and HEK293T cells (human fetal kidney epithelial cell line) were cultured in Dulbecco's modified Eagle medium (DMEM) (Invitrogen-Gibco, Breda, The Netherlands) complemented with 10% (v/v) fetal calf serum (Hyclone, Lonan, Utah), 100 IU/ml penicillin, 100 lg/ml streptomycin and 2 mM L-glutamine (Invitrogen-Gibco). Stable firefly luciferase expressing cells were generated by transducing naïve Huh7 cells with a lentiviral vector expressing the firefly luciferase gene under control of the human phosphoglycerate kinase (PGK) promoter (LV-PGK-Luc). For visualisation of autophagy formation, Huh7 cells were transduced with lentiviral vector expressing the GFP-LC3 fusion protein.

Gene knockdown by lentiviral vector delivered short hairpin RNA (shRNA)

Lentiviral vectors (Sigma–Aldrich), targeting mTOR, 4E-BP1 or GFP (shCon), were obtained from the Erasmus Center for Biomics and produced in HEK 293T cells as previously described [16]. After a pilot study, the shRNA vectors exerting optimal gene knockdown were selected. These shRNA sequences were described in Supplementary Table 1.

To generate stable gene knockdown cells, Huh7 cells were transduced with lentiviral vectors. Since the vectors also express a puromycin resistance gene, transduced cells were subsequently selected by adding 2.5 μg/ml puromycin (Sigma) to the cell culture medium. For the infectious model, HEV particles were incubated with knockdown and control Huh7 cells.

Measurement of luciferase activity

For Gaussia luciferase, the activity of secreted luciferase in the cell culture medium was measured using BioLux Gaussia Luciferase Flex Assay Kit (New England Biolabs). For firefly luciferase, luciferin potassium salt (100 mM; Sigma) was added to cells and incubated for 30 min at 37 ℃. Both gaussia and firefly Luciferase activity was quantified with a LumiStar Optima luminescence counter (BMG LabTech, Offenburg, Germany).

MTT assay

10 mM 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Sigma) was added to cells seeded in 96-well plates and the cells grow at 37 ℃ with 5% CO2 for 3 h. The medium was removed and 100 μL of DMSO was added to each well. The absorbance of each well was read on the microplate absorbance readers (BIO-RAD) at wavelength of 490 nm. All measurements were performed in triplicates.

Quantitative real-time polymerase chain reaction

RNA was isolated with a Machery-NucleoSpin RNA II kit (Bioke, Leiden, The Netherlands) and quantified using a Nanodrop ND-1000 (Wilmington, DE, USA). cDNA was prepared from total RNA using a cDNA Synthesis Kit (TAKARA BIO INC). The cDNA of HEV and GAPDH were amplified by 40 cycles and quantified with a SYBRGreen-based real-time PCR (MJ Research Opticon, Hercules, CA, USA) according to the manufacturer's instructions. GAPDH was considered as reference gene to normalize gene expression. The HEV primer sequences were 5'-ATTGGCCA GAAGTTGGTTTTCAC-3' (sense) and 5'-CCGTGGCTATAATTGTGGTCT-3' (antisense), and the primers of housekeeping gene GAPDH were 5'-TGTCCCCACCCCCAATGT ATC-3' (sense) and 5'-CTCCGATGCCTGCTTCACTACCTT-3' (antisense).

Western blot assay

Proteins in cell lysates were heated 5 min at 95 ℃ followed by loading onto a 10–15% sodium dodecyl sulphate-polyacrylamide gel and separated by electrophoresis (SDS-PAGE). After 90 min running at 120 V, proteins were electrophoretically transferred onto a polyvinylidene difluoride (PVDF) membrane (Invitrogen) for 1.5 h with an electric current of 250 mA. Subsequently, the membrane was blocked with 2.5 ml blocking buffer and 2.5 ml PBS containing 0.05% Tween 20 (PBS-T). It was followed by incubation with rabbit LC3-I/II, p-PKB, p-mTOR, mTOR 4E-BP1, p-4E-BP1 or p-S6 (1:1000) antibody overnight at 4 ℃. The membrane was washed 3 times followed by incubation for 1.5 h with anti-rabbit or anti-mouse IRDye-conjugated secondary antibodies (LI-COR Biosciences, Lincoln, USA) (1:5000) at room temperature. Blots were assayed for actin content as standardisation of sample loading, scanned, and quantified by Odyssey infrared imaging (Li-COR Biosciences, Lincoln, NE, USA). Results were visualised with Odyssey 3.0 software.

Confocal laser electroscope assay

Lipidated LC3 (LC3-II) is a robust marker of autophagic membranes. Autophagosomes were visualised as bright green fluorescent protein GFP-LC3-II puncta by fluorescence microscopy. For nutrient starvation, cells were incubated in EBSS medium with 1 mM pepstatin A and E-64-d solution overnight prior to fix for confocal laser electroscope analysis. The cells were fixed with 70% ethanol and GFP-LC3-II puncta was detected using confocal electroscope.

Statistical analysis

All results were presented as mean ± SD. Comparisons between groups were performed with Mann-Whitney test. Differences were considered significant at a p value less than 0.05.

Results

mTOR inhibition by rapalogs facilitates HEV replication

The 7.2-kb genome of HEV is a single strand positive-sense of RNA containing three overlapping reading frames (ORFs). We employed a model, in which human hepatoma cells

(Huh7) were transfected with a 3' subgenomic construct of the HEV coding sequence, in which the 5' portion of ORF2 was replaced with the in-frame secreted form of luciferase derived from the marine copepod *Gaussia princeps* (p6-luc) (Supplementary Figure 1). Accumulation of luciferase in Huh7 cells thus serves as reporter for HEV RNA synthesis, whereas the loss of the capsid protein in the model system precludes the formation of novel viral particles [10,11]. In parallel, Huh7 cells constitutively expressing a nonsecreted firefly luciferase are used for normalisation of non-specific effects on luciferase signals. In addition, a Huh7 based full length infectious HEV model (p6) was also employed (Supplementary Figure 1) [10,11].

Direct investigation of the phosphorylation status of phospho-Ser-240/224 S6 and phospho-Ser-473 PKB showed that Huh7 cells represent a PI3K-PKB-mTOR-proficient model system (Figure 1A and B). Importantly, inhibiting mTOR rendered this system sensitive to HEV infection, as evident from higher levels of *G. princeps* luciferase, which increased over time. Treatment with 100 and 1000 ng/ml of rapamycin for 48 h resulted in a 1.9 ± 0.4 (mean ± SD, n = 3, $p < 0.05$) and 2.7 ± 0.6 (mean ± SD, n = 3, $p < 0.01$) -fold increase of HEV luciferase activity (Figure 1C) and corresponds to a concomitant decrease in mTOR activity as assessed by phospho-Ser-240/224 S6 levels (Figure 1A). At 72 h, HEV luciferase activity was further increased up to 3.8 ± 0.5 (mean ± SD, n = 3, $p < 0.01$) and 4.9 ± 0.5 (mean ± SD, n = 3, $p < 0.01$) -fold, respectively (Figure 1C). A possible artefact here could be due to the direct growth-promoting effects of rapamycin, but the MTT assay showed that rapamycin did not promote cell growth (Figure 1D).

Next to rapamycin, everolimus is often used for clinical mTOR inhibition following orthotropic organ transplantation. Like rapamycin, everolimus also remarkably permitted HEV replication. In the p6-Luc model, treatment with 1 ng/ml of everolimus has already significantly increased HEV luciferase activity. Treatment with 100 and 1000 ng/ml of everolimus resulted in 7.0 ± 2.2 (mean ± SD, n = 3, $p < 0.01$) and 6.7 ± 1.4 (mean ± SD, n = 3, $p < 0.01$) -fold increase at 48 h, 5.3 ± 0.8 (mean ± SD, n = 3, $p < 0.01$) and 5.6 ± 1.9 (mean ± SD, n = 3, $P < 0.05$) -fold increase of HEV luciferase activity at 72 h (Figure 1E). Everolimus also did not promote cell proliferation determined by MTT assay (Figure 1F). Dephosphorylation of S6 was also confirmed (Figure 1B).

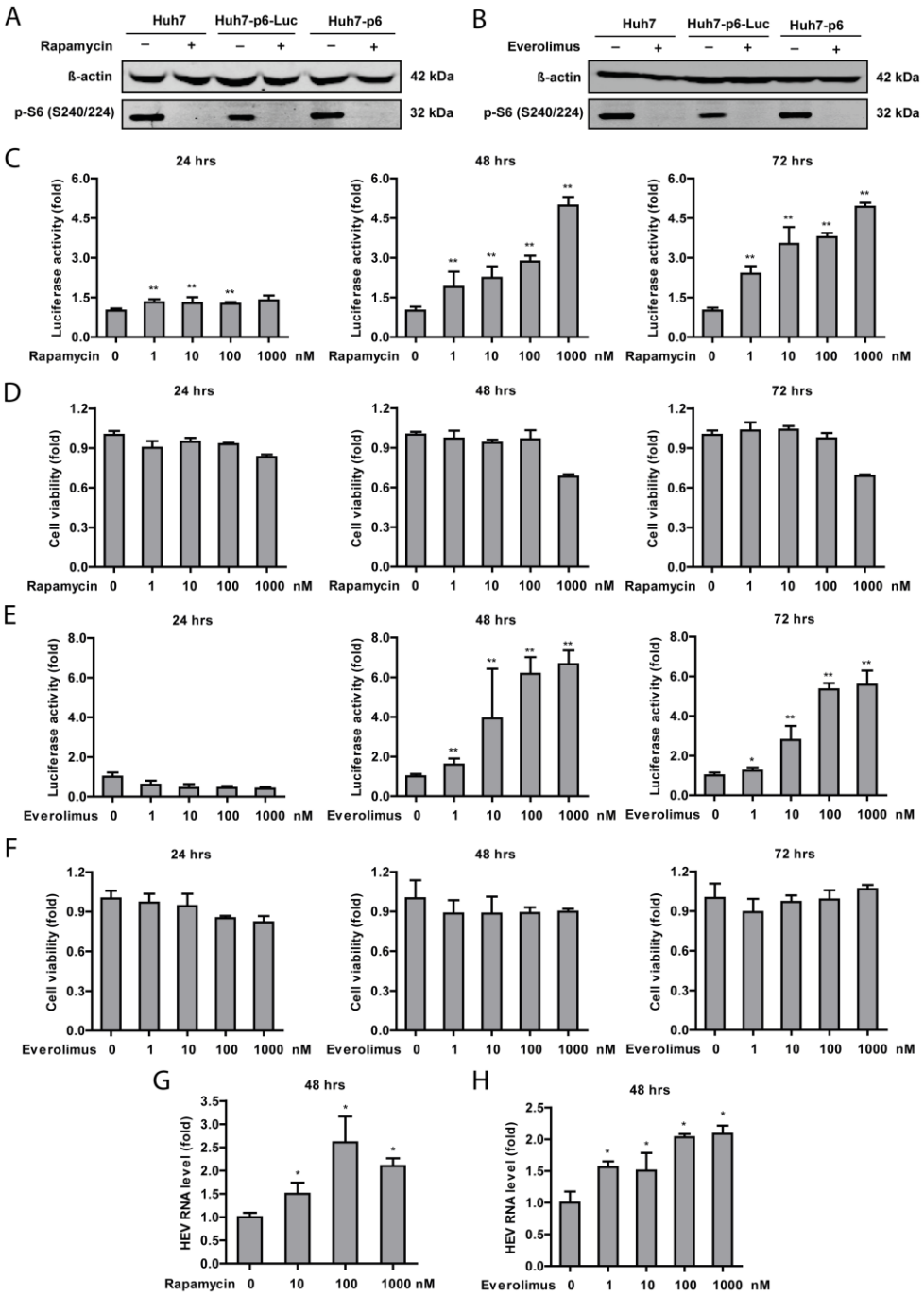

Figure 1. mTOR inhibition by rapalogs facilitate HEV replication. (A) Western blot showing inhibition of S6 phosphorylation by treatment with 500 nM rapamycin for 48 h. β-actin served as an internal reference. (B) Western blot showing inhibition of S6 phosphorylation by treatment with 500 nM everolimus for 48 h. β-actin served as an internal reference. (C) In the Huh7 cell-based subgenomic HEV replicon, treatment with rapamycin dose-dependently increased viral replication-related luciferase activity (mean ± SD, n = 3 independent experiments with each 2–3 replicates). (D) Rapamycin did not increase cell proliferation determined by MTT assay (OD490 value) (mean ± SD, n = 5). (E) In the Huh7 cell-based subgenomic HEV replicon, treatment with everolimus dose-dependently increased viral replication-related luciferase activity (mean ± SD, n = 3 independent experiments with each 2–3 replicates). (F) Everolimus did not increase cell proliferation determined by MTT assay (OD490 value) (mean ± SD, n = 5). (G) In the HEV infectious model, (G) rapamycin (mean ± SD, n = 5) as well as (H) everolimus (mean ± SD, n = 3–6) significantly increased cellular viral RNA determined by qRTPCR. * $p < 0.05$; ** $p < 0.01$.

To exclude that this effect is a consequence of the loss of the capsid protein and ORF3 protein in our luciferase model, we repeated the experiments with the full-length infectious HEV model. Again, HEV infection was facilitated under mTOR-deficient conditions. For instance, treatment with 100 or 1000 ng/ml rapamycin has increased viral RNA levels up to 2.6 ± 0.6 (mean ± SD, n = 5, $p < 0.01$) or 2.1 ± 0.4 (mean ± SD, n = 5, $p < 0.01$) -fold, respectively (Figure 1G). Treatment with 1, 10, 100, and 1000 ng/ml of everolimus for 48 h resulted in an increase of cellular viral RNA up to 1.6 ± 0.1 (mean ± SD, n = 3, $p < 0.01$), 1.5 ± 0.3 (mean ± SD, n = 6, $p < 0.05$), 2.0 ± 0.1 (mean ± SD, n = 3, $p < 0.01$) and 2.1 ± 0.2 (mean ± SD, n = 3, $p < 0.01$) (Figure 1G). Hence, both major drugs used for clinical mTOR inhibition provoke an altered cellular state in hepatocyte-like cells that allows efficient HEV replication to proceed.

Gene silencing of mTOR by RNAi enhances HEV replication

To evaluate the direct effects of mTOR on HEV, Huh7 cells were transduced with integrating lentiviral vectors expressing short hairpin RNA (shRNA) specifically targeting mTOR or a control shRNA (shCon). As shown in Figure 2A, three of the four tested shRNA vectors targeting mTOR exert potent gene silencing capacity, resulting in a profound downregulation of mTOR protein level but an elevation of PKB expression (probably due to a feedback activation). Correspondingly, mTOR silencing resulted in a significant increase of cellular HEV RNA, which was measured by qRTPCR after inoculation of cell culture produced infectious HEV particles for 72 h. For instance, knockdown of mTOR by the shmTOR clone 2 led to 2.6 ± 0.8-fold (mean ± SD, n = 3, $p < 0.05$) increase of HEV RNA (Figure 2A). These data provide direct and strong evidence that mTOR plays an important role in restricting HEV infection.

mTOR limits HEV replication via 4E-BP1

mTOR is a key kinase controlling cellular behaviour. Its most important effector pathways include induction of protein transcription via the p70 S6 kinase pathway (Figure 2B) [15]. However, this pathway does not seem to be a major effector mechanism as inhibition of p70 S6 kinase by its inhibitor PF-478671 did not affect HEV infection (Supplementary Figure 2). Inhibition of another downstream target of mTOR, hypoxia-inducible factor-1alpha (HIF-1a) by FG-4592 (Supplementary Figure 3) also did not affect HEV infection.

Notably, mTOR is also the main inhibitor of autophagy in cellular metabolism and it is possible that HEV replication requires autophagosome formation. However, inhibition of mTOR did not change the levels of microtubule-associated protein 1 light chain 3 b (LC3-II) in our model system (Figure 2C), a hallmark of autophagosome formation. Furthermore, Huh7 cells stably intergraded with a lentiviral vector expressing GFP-LC3-II were used to visualise autophagosome formation. In the positive control groups, cells were either treated with 30 µM chloroquine or under condition of starvation in EBSS medium. As expected, green puncta of LC3-II were clearly emerging, indicating the formation of autophagosomes (Figure 2D). In contrast, no changes of the autophagy machinery were observed with treatment of rapamycin or everolimus (Figure 2D), which was consistent with the results of Western blot (Figure 2C). Thus, these findings exclude the possibility that the proviral effect of rapamycin/everolimus is via the autophagy machinery.

4E-BP1 is another important element induced by mTOR for cellular cap-dependent translation [17]. Treatment with the mTOR inhibitor everolimus (500 nM) for 48 h resulted in clear dephosphorylation of 4E-BP1 as shown by Western blot (Figure 2E). To further confirm the regulation of HEV replication by 4E-BP1, Huh7 cells were transduced with integrating lentiviral vectors expressing shRNA specifically targeting 4E-BP1 or a control shRNA (shCon). Cells stably transduced with the vector were also selected and expanded by adding puromycin to the relevant cell cultures. Four out of five shRNA vectors targeting 4E-BP1 exert gene silencing capacity, resulting in downregulation of total 4E-BP1 protein (Figure 2F). Correspondingly, we selected two 4E-BP1 silencing cell-lines with optimal gene silencing potency that resulted in a significant increase of cellular HEV RNA level, which was measured by qRT-PCR of HEV RNA. For instance, knockdown of 4E-BP1 led to a 1.7 ± 0.6-fold (mean ± SD, $n = 5$, $p < 0.01$) (by the sh4E-BP1 clone 53) and 2.4 ± 0.9-fold (mean ± SD, $n = 4$, $p < 0.05$) (by the clone 56) increase of HEV RNA, respectively (Figure 2G). Consistently, clone 54, with minimal gene silencing efficacy, only exerted a minor effect (1.3 ± 0.3-fold, mean ± SD, $n = 4$, $p > 0.05$) on HEV replication (Figure 2G). These data indicated that the antiviral effect of mTOR is via its downstream target, 4E-BP1.

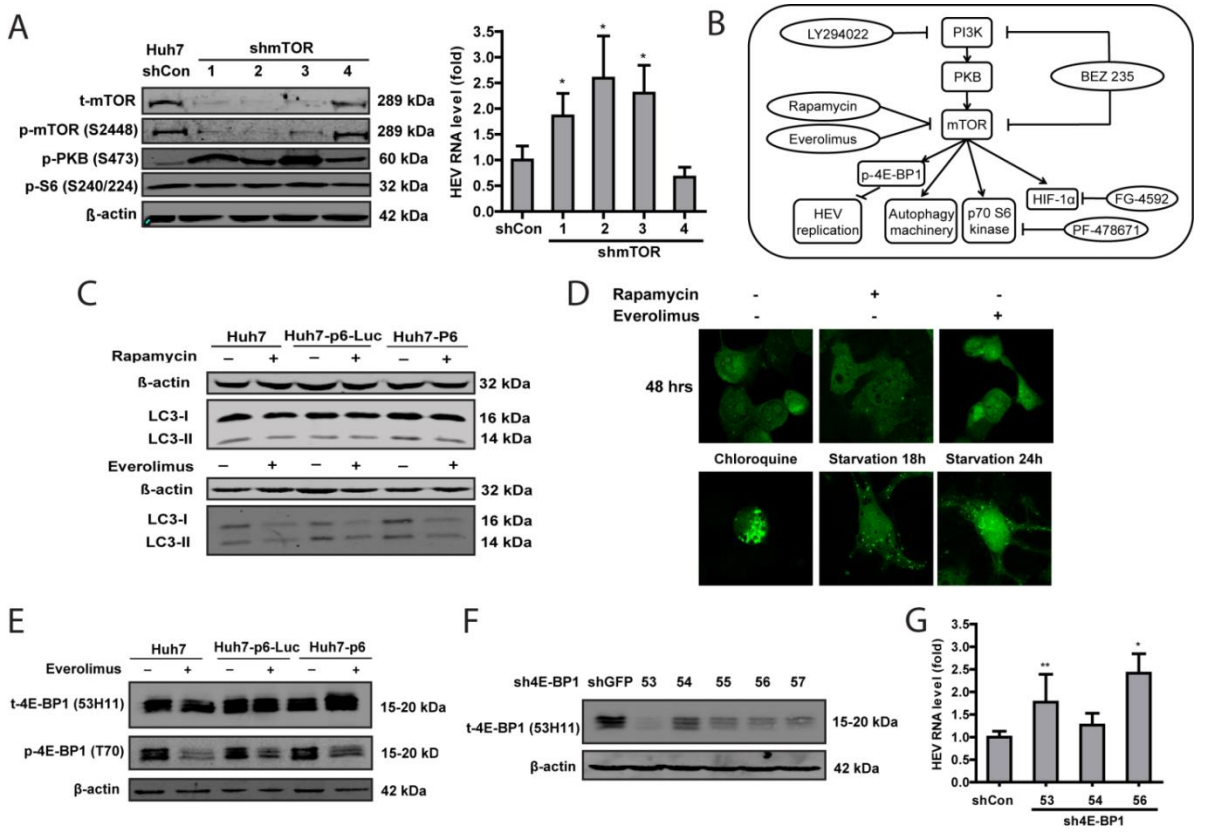

Figure 2. Gene silencing of mTOR and 4E-BP1 by lentiviral RNAi enhances HEV replication independent of autophagy machinery. (A) Knockdown of mTOR by lentiviral shRNA vectors. Compared with the control vector transduced cells, the shmTOR clones 1, 2, and 3 but not 4 exert potent silencing capability shown at protein levels of both total- (t-mTOR) and phospho-mTOR (p-mTOR), which also resulted in dramatic elevation of phospho-PKB (p-PKB). S6 phosphorylation (p-S6) was also determined by Western blot and β-actin served as an internal reference. Correspondingly, knockdown of mTOR resulted in significant increase of cellular HEV RNA level (mean ± SD, n = 3), which were measured by qRT-PCR after inoculation of cell culture produced infectious HEV particles for 72 h. * $p < 0.05$; ** $p < 0.01$. (B) Illustration of the effects on HEV infection by inhibiting different components of the PI3K-PKB-mTOR pathway. Rapamycin/everolimus, inhibitors of mTOR; LY294022, an inhibitor of PI3K-PKB; BEZ-235, a dual inhibitor of PI3K-PKB and mTOR; PF-4708671, an inhibitor of p70 S6 kinase and FG-4592, an inhibitor of HIF-1a were used. (C) Naïve Huh7, subgenomic HEV replicon and HEV infected Huh7 cells were treated with rapamycin and everolimus for 48 h. The accumulation of LC3-II, a hallmark of autophagy formation, was not observed by Western blot analysis. β-actin was served as an internal reference. (D) Consistently, green puncta formation, an indication of autophagosome formation, was not observed in Huh7 cells expressing GFP-LC3-II fusion protein, by treatment of rapamycin and everolimus for 24, 48, and 72 h. In contrast, autophagosome formation was observed in the positive control groups treated with 30 μM chloroquine for 48 h or at the circumstance of starvation in BESS media with 1 mM pepstatin A and E-64-D for either 18 or 24 h. Oil-lenses (40 ×) was used (1024 × 1024 image). (E) Western blot showed inhibition of 4E-BP1 phosphorylation by treatment of 500 nM everolimus for 48 h. β-actin served as an internal reference. (F) Knockdown of 4E-BP1 by lentiviral shRNA vectors. Compared with the control vector transduced cells, the sh4E-BP1 clone 53, 55, 56, and 57 but not 54 expert potent silencing efficacy shown at protein levels of total 4E-BP1 (t-4E-BP1), β-actin served as an internal reference. (G) Correspondingly, knockdown of 4E-BP1 resulted in significant increase of cellular HEV RNA level (mean ± SD, n = 5). * $p < 0.05$; ** $p < 0.01$. (This figure appears in colour on the web.)

Inhibition of PI3K-PKB promotes viral replication

Although distinct molecules, rapamycin and everolimus share important structural characteristics. To exclude the possibility that the effects of these compounds on HEV replication represent a mTOR-independent off-target effect, independent confirmation of the role of PI3K/PKB/mTOR signaling cascade in preventing HEV replication was sought through experiments, in which more upstream elements of this signaling cascade were targeted (Figure 2B). When Huh7 p6-Luc cells were treated with different concentrations (0.1–10 μM) of the well-established PI3K inhibitor LY294002, enhancement of HEV replication became apparently similar to that observed with mTOR inhibitors (Figure 3A), which was also not related to enhanced cell proliferation (Figure 3B). Consistently, LY294002 also significantly increased cellular HEV RNA in the infectious model up to 3.2 ± 1.1-fold (mean ± SD, n = 3, $p < 0.05$) at a dose of 10 μM (Figure 3C). These effects corresponded to the observed inhibition of the biological target (Figure 3D).

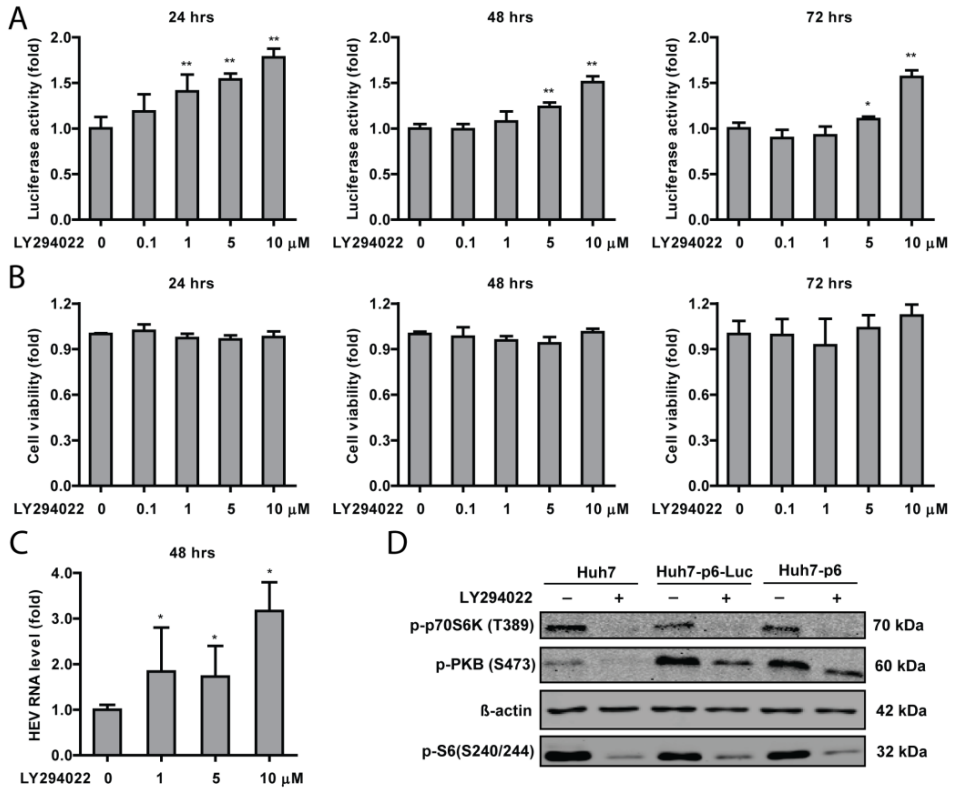

Figure 3. Inhibition of PI3 K-PKB promotes viral replication. (A) In the Huh7 cell-based subgenomic HEV replicon, treatment with LY294022, a PI3K inhibitor, dose dependently increased viral replication-related luciferase activity (mean ± SD, n = 3). (B) LY294022 did not affect cell proliferation determined by MTT assay (OD490 value) (mean ± SD, n = 4). (C) In the HEV infectious model, LY294022 significantly increased cellular viral RNA determined by qRT-PCR (mean ± SD, n = 3). (D) Western blot showed inhibition of PKB, S6 and p70 S6 kinase phosphorylation by treatment of 5 lm LY294022 for 48 h. β-actin served as an internal reference. Treatment time was indicated as 24, 48 or 72 h. * $p < 0.05$; ** $p < 0.01$.

Simultaneous inhibition of PI3K and mTOR further enhanced viral replication

Simultaneous treatment with rapamycin/everolimus and LY294002 apparently had stronger effects than rapamycin or LY294002 alone. The strongest effect on HEV replication was observed with the combination of 100 ng/ml rapamycin and 10 µM LY294002 at 48 h (up to 12.1 ± 3.1-fold, mean ± SD, n = 11, $p < 0.01$ vs. untreated; $p < 0.01$, vs. rapamycin; $p < 0.01$, vs. LY294002) (Figure 4A), and at 72 h with the combination of 1000 ng/ml rapamycin and 10 µM LY294002 (up to 31.7 ± 9.9-fold, mean ± SD, n = 11, $p < 0.01$ vs. untreated; $p < 0.05$, vs. rapamycin; $p < 0.01$, vs. LY294002) (Figure 4A). Similar effects were observed when everolimus was combined with LY294002 (Figure 4B). Furthermore, these results were found not to be related to enhancement of cell proliferation either with rapamycin (Supplementary Figure 4A) or everolimus (Supplementary Figure 4B). BEZ-235 is a dual inhibitor of mTOR and PI3K signalling, which is at the stage of clinical development for treating cancer patients (NCT00620594, ClinicalTrials.gov) (Figure 2B). We further investigated the effect of simultaneously inhibiting PI3K-PKB and mTOR by a single compound BEZ-235. As shown in Figure 4C and D, BEZ-235 significantly promoted HEV infection in both models. Furthermore, results corresponded to inhibition of biological targets of this pathway (Figure 4E). The most straightforward interpretation of these data is that HEV can efficiently replicate in the context of deficient signaling through the PI3K-PKB-mTOR cascade.

Discussion

Large zoonotic reservoirs of hepatitis E exist in cattle and poultry and it is generally accepted that humans are frequently infected with the virus [7]. Almost invariably, however, the disease remains subclinical [2]. Here we present evidence that the inability of HEV to effectively replicate in humans is linked to constitutive mTOR activation. This novel action of mTOR in directly counteracting viral replication in liver cells themselves rather than acting through the adapted immune system, represents a highly novel non-canonical action of this kinase in a new adapted immune system-independent antiviral mechanism and thus our results are highly unexpected.

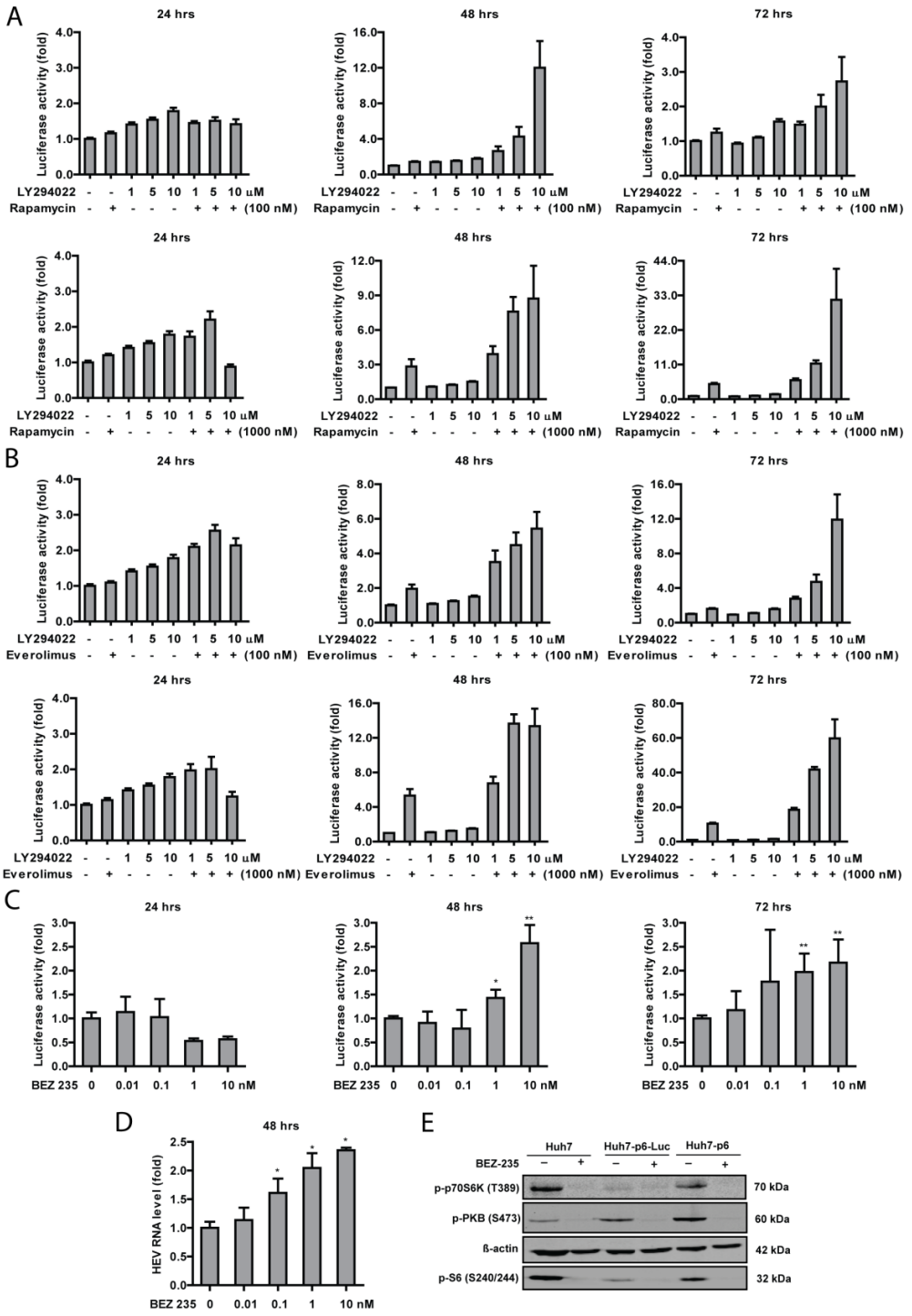

Figure 4. Simultaneous inhibition of PI3K and mTOR further increased viral replication. In the HEV subgenomic replicon, viral replication-related luciferase activity was present when 1, 5 or 10μMLY294022 was combined with 100/1000 nM of rapamycin (A) or everolimus (B). Treatment time was indicated as 24, 48 or 72h. Data was presented as mean ± SD, n = 11 replicates in total. (C) BEZ-235 is a dual inhibitor of PI3K-PKB and mTOR. In the Huh7 cell-based subgenomic HEV replicon, treatment with BEZ-235 significantly increased viral replication-related luciferase activity (mean ± SD, n = 5). (D) In the HEV infectious model, BEZ-235 significantly increased cellular viral RNA determined by qRT-PCR (mean ± SD, n = 3). (E) Western blot showed inhibition of PKB, S6 and p70 S6 kinase phosphorylation by treatment of 1 nM BEZ-235. β-actin served as an internal reference. Treatment time was indicated as 24, 48 or 72 h. * $p < 0.05$; ** $p < 0.01$.

Patients after orthotropic organ transplantation when receiving immunosuppressants, such as rapalogs, to prevent rejection are well known to be at extremely high risk of Patients after orthotropic organ transplantation when receiving immunosuppressants, such as rapalogs, to prevent rejection are well known to be at extremely high risk of developing chronic hepatitis with persistence of infection [3,6,18]. It is known that the clinical symptoms of this hepatitis reacts very well to reducing dose of immunosuppression [6]. Hitherto, this beneficial effect of decreasing immunosuppressive therapy was attributed to increased immunity [19]. However, different types of immunosuppressants can also have direct effects on HEV replication in the target cells of the virus [20]. In transplantation patients, the blood concentrations of rapalogs can reach approximately 15 ng/ml [21,22], whereas the levels in cancer patients can be up to approximately 100 ng/ml [23]. We have demonstrated that 1 ng/ml everolimus was sufficient to trigger significant stimulatory effects on HEV replication *in vitro*, which thus clearly bears important clinical relevance.

In fact, more evidence supporting the potential proviral effects of rapalogs have come from hepatitis B virus (HBV) infected patients. In a randomised clinical trial comparing two everolimus dosing schedules in patients with advanced hepatocellular carcinoma, four patients were hepatitis B surface antigen (HBsAg)-seropositive. During treatment of everolimus, all these patients experienced episodes of HBV flare with >1-log increase in the serum HBV DNA level accompanied by alanine transaminase elevations [24]. Similarly, a patient with renal cell carcinoma also experienced a HBV flare during everolimus treatment [25]. These observations could be explained by affecting the adaptive immune system but may also by direct effects on viral replication. The current experimental study has firmly demonstrated the proviral effects of both rapamycin and everolimus in two state-of the- art HEV cell culture models. Further detailed mechanistic investigation has revealed an antiviral function of the PI3K-PKBmTOR pathway, which appears to support the recent clinical observations in viral hepatitis patients [18,24,25].

Another group of patients at high risk for HEV caused death constitute pregnant women [26]. Although this effect is in literature generally linked to diminished immunity [26], immune suppression during pregnancy is relatively moderate [27]. Interestingly, the increased nutritional demands of pregnancy [28] provoke a powerful activation of the ATP/ADP-sensitive kinase AMPK [29]. In turn, this kinase is a potent inhibitor of mTOR [30] and indeed pregnancy is

associated with a significant downregulation of mTOR [31]. It is tempting to speculate that pregnancy-specific downregulation of mTOR may help to understand why this group is specifically sensitive to HEV infection. In apparent agreement, malnutrition in general is also associated with susceptibility to HEV [8]. We thus speculate that HEV may preferentially affect the human population when hepatic mTOR activity is below its constitutive level.

Because of its favourable side-effect profile, rapalog therapy is quickly gaining popularity for treating a variety of clinical syndromes, especially in oncological disease, in congenital diseases like the Peutz-Jeghers syndrome and the Tuberous sclerosis complex, in transplantation medicine and autoimmunity. Therefore, recognition of the anti-HEV function of PI3K-PKB-mTOR pathway bears magnificent implications in clinical practice regarding the choice of particular immunosuppressant for HEV-infected organ transplant recipients. In particular, the use of mTOR inhibitors in these patients should be taken with caution. In addition, these results may also help to understand the underlying mechanism why pregnant women are more susceptible to HEV infection with devastating outcome.

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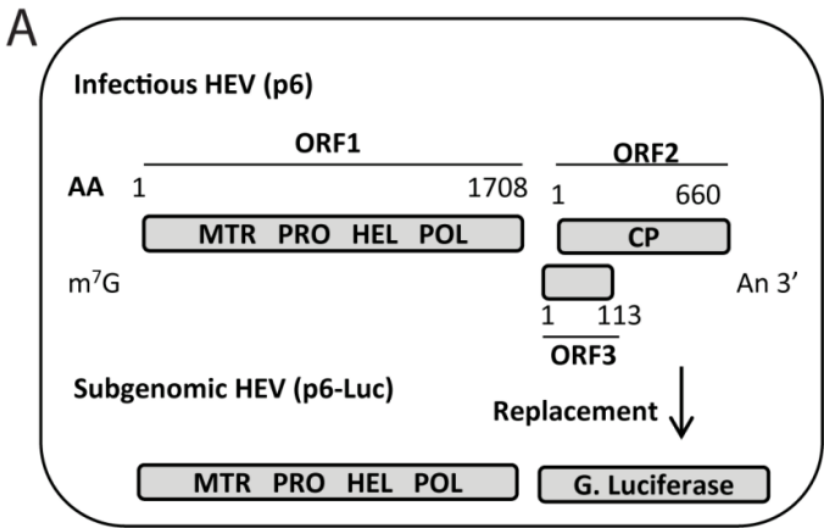
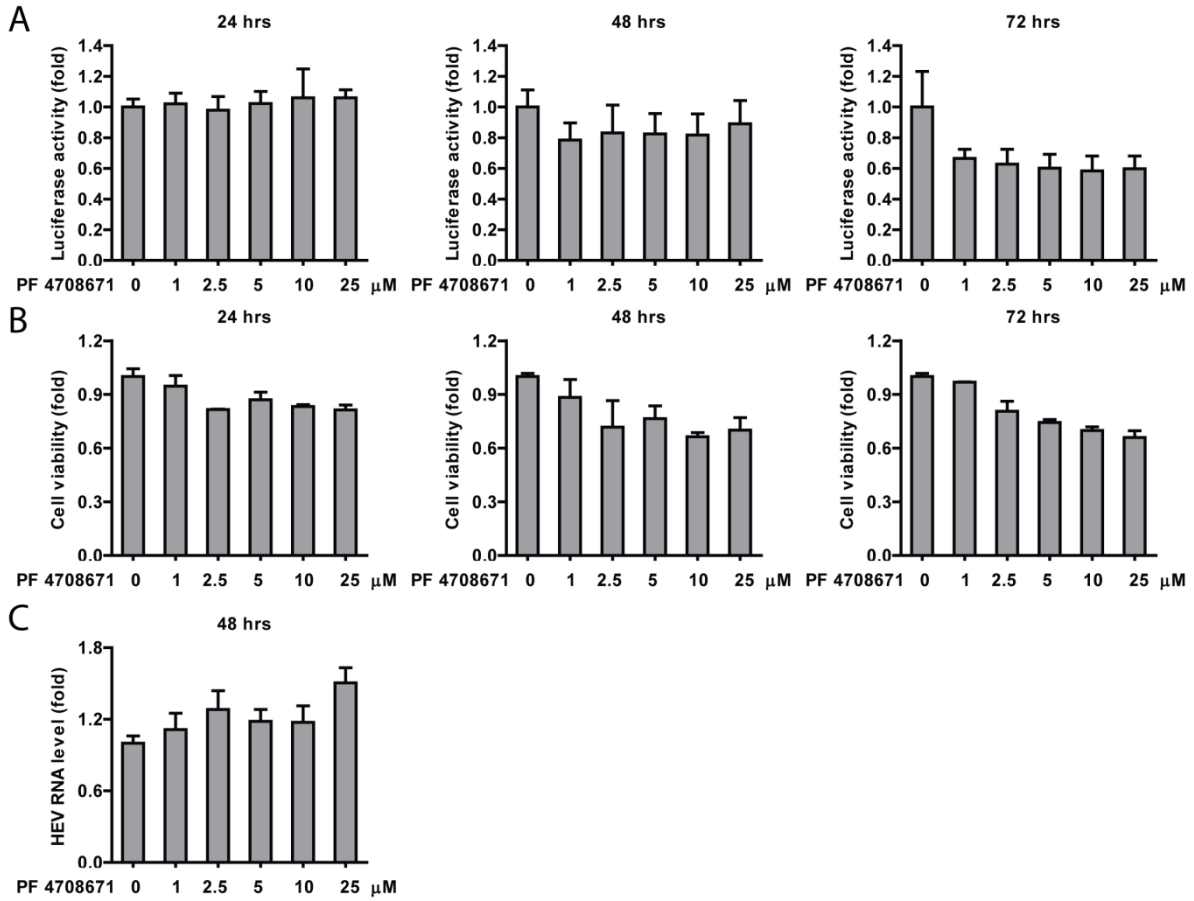
Supplementary Materials

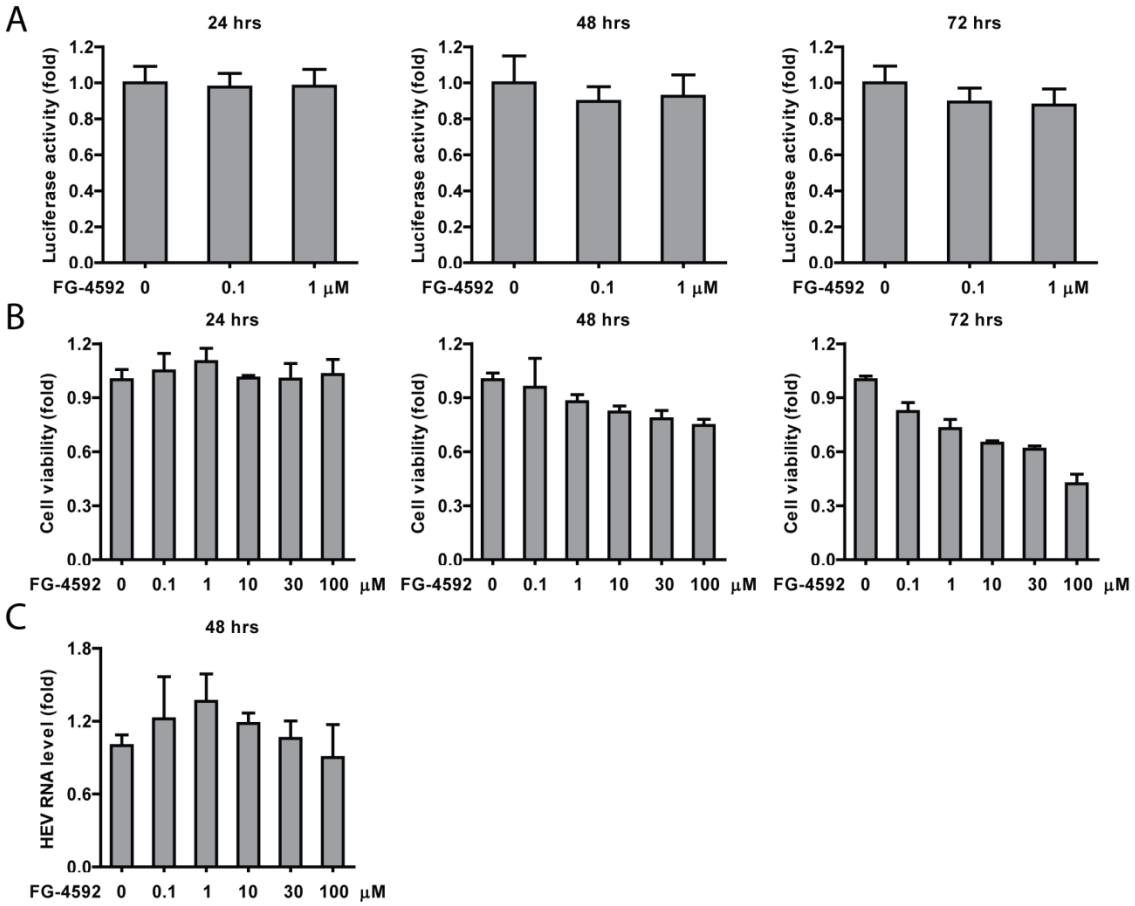
Supplementary Figures and Tables



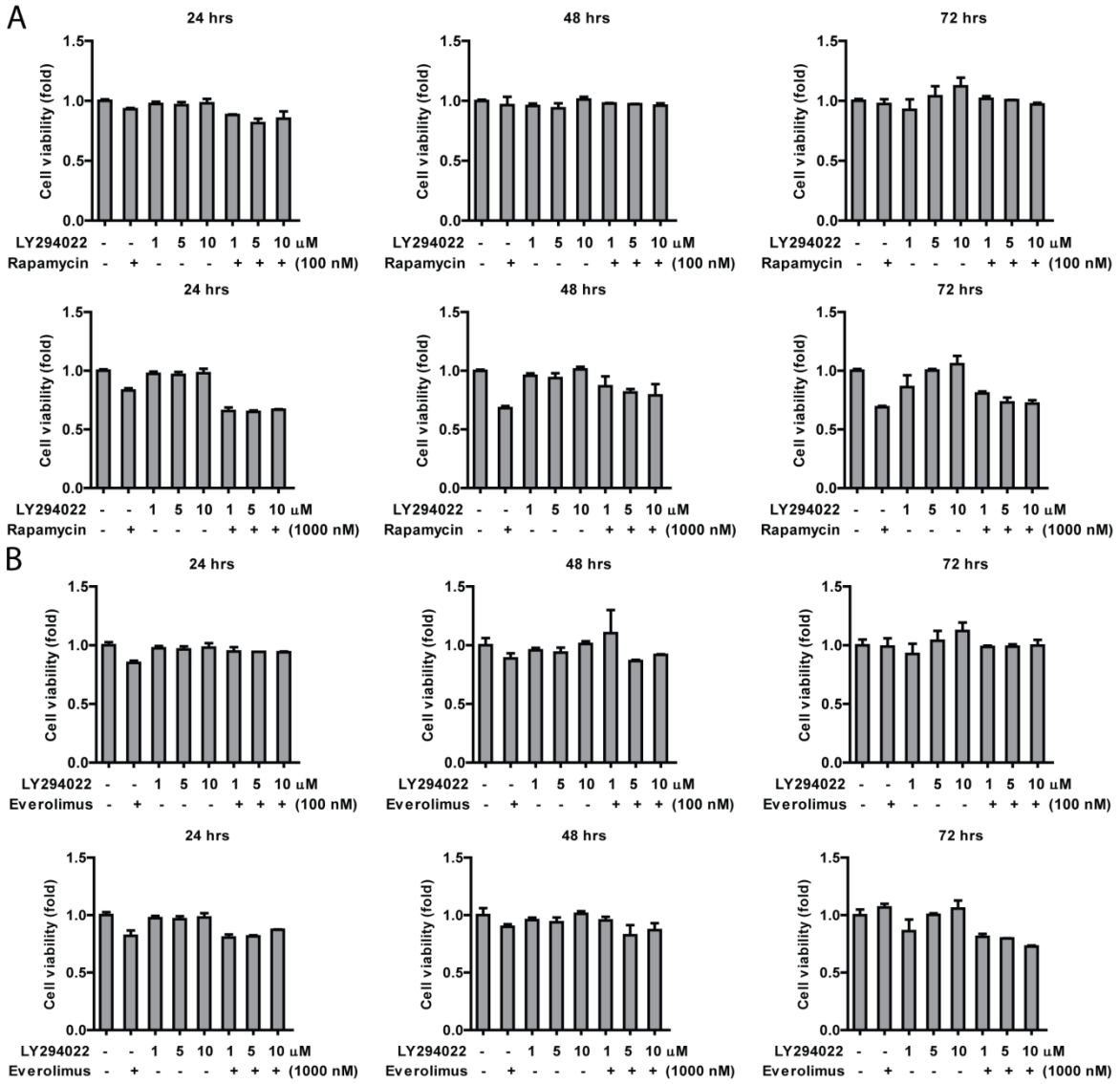
Supplementary Figure 1. Genomic structure of the infectious HEV (p6) and subgenomic HEV replicon (p6-Luc) models.



Supplementary Figure 2. PF-478671, a p70 S6 kinase inhibitor has no effect on HEV replication. (A) In the Huh7 cell-based subgenomic HEV replicon, treatment with PF-478671 for 24, 48 and 72 hrs did not have clear effects on viral replication-related luciferase activity. (B) PF-478671 did not increase cell proliferation determined by MTT assay (relative OD_{490} value). (C) In the HEV infectious model, treatment with PF-478671 also did not have clear effects on HEV cellular RNA.



Supplementary Figure 3. FG-4592, a HIF-1α inhibitor has no effect on HEV infection. (A) In the Huh7 cell-based subgenomic HEV replicon, treatment with FG-4592 for 24, 48 and 72 hrs did not have clear effects on viral replication-related luciferase activity. (B) FG-4592 did not increase cell proliferation determined by MTT assay (relative OD_{490} value). (C) In the HEV infectious model, treatment FG-4592 also did not have clear effects on HEV cellular RNA.



Supplementary Figure 4. Simultaneous inhibition of PI3K and mTOR did not affect cell proliferation. In the HEV subgenomic replicon, viral replication-related luciferase activity was presented when 1, 5 or 10 μM LY294022 was combined with 100/1000 nM of rapamycin (A) or everolimus (B). Treatment time was indicated as 24, 48 or 72 hrs. Cell proliferation determined by MTT assay (relative OD_{490} value).

Supplementary Table 1. shRNA sequences

shmTOR-1	CCGGGCCAGAATCTATTCATTCTTTCTCGAGAAAGAATGAATAGATTCTGGCTTTTTG
shmTOR-2	CCGGGCCTTGTTTGTGGCTCTGAATCTCGAGATTCAGAGCCACAAACAAGGCTTTTTG
shmTOR-3	CCGGGAACCAATTATACCCGTTCTTCTCGAGAAGAACGGGTATAATTGGTTCTTTTTG
shmTOR-4	CCGGGCTGTGCTACACTACAAACATCTCGAGATGTTTGTAGTGTAGCACAGCTTTTTG
sh4E-BP1-53	CCGGGCCAGGCCTTATGAAAGTGATCTCGAGATCACTTTCATAAGGCCTGGCTTTTTG
sh4E-BP1-54	CCGGAGGATCATCTATGACCGGAAACTCGAGTTTCCGGTCATAGATGATCCTTTTTTG
sh4E-BP1-55	CCGGACAGTTTGAGATGGACATTTACTCGAGTAAATGTCCATCTCAAACTGTTTTTTG
sh4E-BP1-56	CCGGCGGTGAAGAGTCACAGTTTGACTCGAGTCAAACTGTGACTCTTCACCGTTTTTG
sh4E-BP1-57	CCGGGCGCAATAGCCCAGAAGATAACTCGAGTTATCTTCTGGGCTATTGCGCTTTTTG

CHAPTER 4

Chronic Hepatitis E in Solid-organ Transplantation: the Key Implications of Immunosuppressants

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Purpose of review

Solid-organ recipients infected with hepatitis E virus (HEV) bear an extremely high risk of developing chronic hepatitis, although this virus only causes acute infection in the general population. Immunosuppressive medication universally used after transplantation to prevent organ rejection appears to be a main risk factor for developing chronic infection. This review aims to overview and emphasize the current clinical and experimental evidence regarding the key implications of immunosuppressants in chronic hepatitis E.

Recent findings

Over 60% of organ recipients who are infected with HEV develop chronic hepatitis. Immunosuppressant treatment after transplantation was identified as a key risk factor. Therefore, dose reduction or even withdrawal of immunosuppressants is considered as the first intervention strategy to achieve viral clearance in these patients. Otherwise, ribavirin, as an off-label medication, is considered as an antiviral treatment, with compelling outcomes observed so far. Interestingly, in addition to a common immunosuppression

property that can favour HEV infection in general, different types of immunosuppressants may exert differential impacts on the infection course in patients. Furthermore, potential interaction may exist between particular immunosuppressant and ribavirin. With the recent development of a cell culture system for HEV, experimental research has been initiated to investigate how immunosuppressive drugs interact with HEV infection.

Summary

On the basis of the current evidence, it remains impossible to define an optimal immunosuppressive protocol for these HEV-infected patients. However, the realization of this clinical issue and the initiation of translational research using cell culture models of HEV have been represented as milestones in this field.

Keywords

chronic HEV, clinical evidence, experimental evidence, immunosuppressive medication

Key points

- Over 60% of organ recipients who are infected with HEV develop chronic hepatitis.
- Thus, the universal use of immunosuppressants after transplantation bears the foremost importance in the development of chronic hepatitis E in organ recipients.
- Dose reduction or even withdrawal (if possible) of immunosuppressants is now considered as the first intervention strategy to achieve viral clearance.
- In addition to a common immunosuppressive property, strong evidence from both clinical and experimental research indicates that different types of immunosuppressants could differentially affect the course of HEV infection.
- The recent development of genotype-3-based cell culture models has enabled mechanistic investigation of how immunosuppressants act on HEV infection.

Introduction

Hepatitis E virus (HEV) is one of the most common causes of acute hepatitis worldwide and is emerging as a global health issue. Initially, this infection was only thought to be a health issue in developing countries, caused mainly by the water-transmitted genotype 1 and 2 viruses. Although it is self-limiting and cleaned spontaneously in general, fulminant hepatitis and high mortality are described, reaching 25% in the case of pregnant women [1]. Epidemics affecting thousands of people have periodically occurred in Asia and Africa. In the recent (2012–2013) hepatitis E outbreak amongst refugees in South Sudan, a total of 5080 acute jaundice syndrome cases had been reported and an acute jaundice syndrome case-fatality rate of 10.4% was observed amongst pregnant women [2].

In the Western world, the prevalence of HEV is mainly genotypes 3 and 4 with zoonotic nature, and eating undercooked pork or game products serves as the primary transmission route. HEV infection of healthy individuals almost exclusively remains subclinical, and otherwise causes an acute and self-limiting infection with low mortality rate [3]. However, it is occasionally recognized as a potential cause of decompensation in patients with pre-existing chronic liver disease and can result in a very high mortality [4,5]. In fact, the emerging issues of HEV infection in developed countries are arising from immunocompromised individuals that include HIV patients [6], cancer patients receiving chemotherapy [7] and organ transplantation recipients [8]. In contrast to the initial thought that

HEV only causes acute infection, those immunocompromised patients, with solid-organ transplantation recipients as the most representative population, appear to have extremely high risk of developing chronic hepatitis [9].

Despite the under diagnosis and lack of indepth mechanistic investigation, the use of immunosuppressants after transplantation is conceivably a key determinant of developing chronic hepatitis in HEV-infected recipients. In addition to a general suppressive effect on the immune system that is crucial for controlling viral infection by the human body, different types of immunosuppressive drugs may also exert differential interactions with the virus, resulting in either favouring or restricting the infection [10]. Therefore, we aim to provide a comprehensive overview and indepth emphasis regarding the role of immunosuppressive medication in the development of chronic hepatitis E in solid-organ transplantation. With substantial evidence from both clinical practice and experimental investigations, we hope that this article can serve as an important reference for the choice of an optimal immunosuppressive protocol for solid-organ recipients who are infected with HEV.

Chronic hepatitis E in solid-organ transplantation

Chronic hepatitis is an ongoing process that causes injury to the cells of the liver, with inflammation which lasts for longer than 6 months. The commonly used tests for diagnosing HEV infection include detection of IgM and IgG anti-HEV antibodies, and detection of HEV RNA in human serum and plasma [11]. In general, IgM antibody is detectable only for 3–12 months, whereas IgG antibody persists for many years. As the presence of HEV RNA indicates ongoing infection, the current definition of chronic hepatitis E is based on the positivity of HEV RNA for more than 6 months [3].

Although persistent infection was reported in cases a decade ago [12,13], HEV was considered as an agent resembling hepatitis A that causes acute hepatitis only [14], until then an unexpected finding that 8 out of 14 HEV-infected organ transplant recipients developed chronic hepatitis. Subsequently, a series of studies have confirmed the existence of chronic hepatitis E in various organ transplantation settings, including liver [15–20], kidney [15,18,21,22], heart [18,23,24], lung [18,25] and multiorgan transplantation [18]. Estimated from the series of published cohort studies (mainly from western countries), the prevalence of anti-HEV IgG is

approximately 11.6% and genomic viral RNA is 2% in solid-organ transplant patients. A total of 65% of patients who were positive for HEV RNA detection developed chronic infection [26].

Chronic hepatitis E in organ recipients is often considered as a result of primary infection, whereas recent evidence suggested that HEV recurrence can also cause chronic hepatitis in transplantation recipients [27]. The current assumption is that it is exclusively caused by genotype 3 HEV, which is prevalent in the industrialized countries. It is zoonotic (animals serve as a reservoir) and is spread mainly through eating undercooked pork or game products [1]. An individual case study reporting a liver transplant recipient who received an organ from an occult HEV-infected donor, developing to chronic HEV infection, convincingly illustrated the transmission can also occur via the transplanted allograft [28]. An intriguing question is whether other genotypes can also cause infection and develop chronic hepatitis in transplantation patients, as current diagnosis of HEV in these patients almost exclusively focus on genotype 3.

Compared with patients with self-limiting infection, chronic HEV-infected patients have evidently a shorter interval time between organ transplantation and diagnosis of liver injury, resulting in a rapid progression to liver fibrosis and cirrhosis. This has been reported in kidney [29], liver [30] as well as heart [31] transplantation, although the exact risk rate is still unclear. In a large retrospective multicentre study, 8 out of 56 HEV-infected organ recipients developed cirrhosis, whereas two liver transplant patients required a second liver transplant and two died of decompensated cirrhosis.

The crucial role of immunosuppressants in chronic hepatitis E

Immunosuppressants are universally used after organ transplantation for life to prevent graft rejection. These medications can modulate viral infection not only by inhibiting host immunity but also by directly affecting the virus life cycle. Current clinical and experimental evidence has supported the crucial role of immunosuppressive drugs in developing chronic hepatitis after HEV infection in organ recipients.

Clinical evidence

Clinically, chronic hepatitis E is almost exclusively associated with immunocompromised conditions caused either by the diseases itself (i.e. HIV infection [6]) or by treatment (i.e. chemotherapy [7] or immunosuppressants [8]). In addition, chronic hepatitis E was also described in a few cases with undefined disease conditions, but those patients conceivably are also immunocompromised [32]. Although a very recent case report suggested the existence of chronic hepatitis E in immunocompetent patients [33], it remains a debate whether this patient with a history of an autoimmune disease can be considered as 'truly' immunocompetent [34]. Thus, the universal use of immunosuppressants after transplantation bears the foremost importance in the development of chronic hepatitis E in organ recipients.

Currently, different types of immunosuppressants with distinct mechanism of action are widely used after transplantation, including corticosteroids, calcineurin inhibitors, mammalian target of rapamycin (mTOR) inhibitors and select antiproliferative agents [35]. These agents can effectively suppress the immune response via direct or indirect mechanism to prevent organ rejection. In addition to their primary task of inhibiting T-cell proliferation, immunosuppressants can also affect the function of other types of immune cells, including B cells, dendritic cells and natural killer cells [36]. As the immune system plays a central role in controlling and even eliminating viral infection, suppression of immune response will thus favour the infection. Extensive clinical evidence has demonstrated the association of immunosuppressive medication with a more aggravated course of hepatitis C virus (HCV) re-infection after liver transplantation as well as more resistance to antiviral therapy [37,38]. Potent immunosuppression is also associated with BK virus reactivation in kidney transplant recipients [39]. Despite the limited study, the current available clinical data has already indicated a strong association between immunosuppressive medication and high risk of chronic hepatitis E in organ transplantation patients [26]. Virtually all types of immunosuppressants can profoundly inhibit T-cell activation and impair HEV-specific T-cell responses in solid-organ transplant patients, which was involved in chronic HEV infection. T-cell responses can appear again after HEV clearance [27]. In addition, attenuation of lymphocytes and CD2, CD3 and CD4 T cells were also reported in organ recipients with persistent infection.

Interestingly, different immunosuppressive regimens may differentially affect the infection course in patients. Steroids have been used in the transplantation setting since the early years. The current knowledge of effect of steroids on HEV infection after transplantation is limited, although one case report has documented a good clinical and biochemical response to steroid therapy in an acute hepatitis E patient with autoimmune hepatitis, who maintained health with low doses of steroids [40]. Tacrolimus is one type of calcineurin inhibitor. A relatively large retrospective study has reported that tacrolimus, but not another type of calcineurin inhibitor cyclosporine A (CsA), is significantly associated with high risk of chronic hepatitis E in organ recipients [26]. Reducing the dosage of tacrolimus was proposed to result in clearance of HEV in cases of kidney transplantation with acute infection [41]. Mycophenolatemofetil (MMF), a pro-drug of mycophenolic acid (MPA), is part of the antiproliferative agents. Recent evidence from heart transplant recipients supported the potential benefits of immunosuppressive treatments containing MMF, which may lead to more frequent HEV clearance [23].

Given the strong association between immunosuppressants and chronic hepatitis E, it is not surprising that withdrawal or dose reduction of immunosuppression is often used as the first intervention strategy to achieve viral clearance in HEV infected organ recipients, despite this approach may bear a high risk of acute rejection [21,42,43]. However, long-term follow-up is required to assess the eventual outcome. This strategy should be used with extra caution in patients at higher risk of rejection or in those who are more difficult to monitor for rejection (e.g. in heart and lung transplantation). Nevertheless, manipulation of immunosuppression does not always result in viral clearance. Although no licensed drug is available, pegylated interferon, ribavirin or a combination are currently used as off-label treatment [44,45]. In particular, ribavirin monotherapy has achieved substantial success in a series of patients with viral clearance [25,46–49]. A recent, retrospective, multicentre study based on 59 solid-organ transplant patients with prolonged HEV viraemia observed HEV clearance in 95% of the patients after ribavirin medication [50]. This is very different from the setting of chronic hepatitis C that ribavirin is generally considered to have little or no detectable antiviral activity as monotherapy in HCV patients [51]. Only when combining with interferon-alpha, ribavirin doubles the sustained virologic response rate, compared with interferon alone [52]. When applying ribavirin, in addition to the side-effect of treatment-

induced low haemoglobin level, potential drug–drug interaction should also be taken caution [48]. In a case series of six heart recipients with chronic hepatitis E, one patient under combination treatment of tacrolimus and everolimus could not clear the virus even after ribavirin treatment, whereas others with tacrolimus combined with prednisolone and MMF cleared the virus spontaneously, after immunosuppression reduction or ribavirin treatment [31]. Thus, it is very important and urgent to properly evaluate the definitive efficacy of ribavirin as well as the potential interaction with immunosuppressants with well-designed clinical trials in chronic hepatitis E patients.

Experimental evidence

Translational and fundamental research of hepatitis E has long been hampered by the lack of efficient cell culture system. The identification of a genotype 3 clone (the exact genotype causing chronic hepatitis) that can propagate in cell culture paved the way towards the establishment of cell culture models [53]. On the basis of this isolate, a subgenomic replicon that can mimic viral replication without viral production and a full-length infectious model have been recently established [54]. With this system that can efficiently model HEV infection in cell culture, the antiviral effects of ribavirin and interferon-alpha have been confirmed *in vitro* [55]. Moreover, the effects and mechanism of action of immunosuppressants on HEV are also being investigated [56,57].

In these cell culture models, HEV infection is not affected by treatment with steroids, but can be enhanced by both the calcineurin inhibitors, CsA and tacrolimus [57]. This is very different from the setting of HCV cell culture system. Studies have reported on the enhancement of HCV entry by steroids [58], no effect of tacrolimus [59,60] and potent antiviral effect of CsA [59]. The cellular targets of CsA, cyclophilin A and B, are the host factors supporting HCV [61,62] but suppress HEV infection [57]. This may explain the dichotomal effects on these two viruses. The proviral effects were also reported with mTOR inhibitors, rapamycin and everolimus [56]. mTOR is a central element within the phosphatidylinositol-3 kinase (PI3K)–protein kinase B–mTOR signalling, and integrates nutritional information and receptor tyrosine kinase signalling to control cellular growth via a variety of cellular effectors, including activation of p70 S6 kinase and subsequent protein synthesis as well as inhibition of autophagy [63]. Autophagy plays an important role in the regulation of viral

infections [64], whereas the stimulatory effects of mTOR inhibitors on HEV were thought to be independent of autophagy machinery [56].

MPA has been extensively demonstrated to be antiviral against a broad spectrum of DNA and RNA viruses, including dengue virus [65], West Nile virus [60], yellow fever virus [66], Chikungunya virus [67] and HCV [60]. Consistently, the antiviral effect of MPA was also observed in the cell culture models of HEV [55,57]. In addition, the combination of MPA and ribavirin resulted in a moderately enhanced antiviral effect against HEV [57]. The antiviral mechanism of MPA was thought to bind and inhibit inosine -5'-monophosphate dehydrogenase enzyme to deplete cellular nucleotide pools. Supplementation of exogenous guanosine in cell culture completely overcomes the anti-HEV activity of MPA, confirming this mechanism of action in the setting of HEV [55,57], whereas this is not in case of HCV infection [68].

Conclusion

Immunosuppressive medication after transplantation is one of the most important risk factors associated with chronic hepatitis in organ recipients who are infected with HEV. Thus, dose reduction or even withdrawal (if possible) of immunosuppression is now considered as the first intervention strategy to achieve viral clearance in these patients. Despite a common immunosuppressive property, strong evidence from both clinical and experimental research indicates that different types of immunosuppressants could differentially affect the course of HEV infection. Calcineurin and mTOR inhibitors may promote but MMF/MPA suppress the HEV infection independent of their general effects on immune system.

Nevertheless, the current clinical studies are not able to conclusively address the impacts of different immunosuppressants, because of limited patient numbers and the lack of long-term follow-up to assess the eventual outcome. On the other hand, the mechanistic investigation has only been recently initiated. Therefore, it is still not sufficient to fully explain the clinical observations by experimental findings. However, the realization of this clinical issue in organ recipients and the development of cell culture models of this virus have paved the way for a flourishing future in this field. Hopefully, it will also promote the initiation of randomized, controlled clinical studies to unravel the differential actions of different immunosuppressants in HEV-infected patients in the near future.

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CHAPTER 5

Ribavirin Inhibits In Vitro Hepatitis E Virus Replication through Depletion of Cellular GTP Pools and Is Moderately Synergistic with Alpha Interferon

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Abstract

Hepatitis E virus (HEV) is a common cause of acute hepatitis that results in high mortality in pregnant women and may establish chronic infections in immunocompromised patients. We demonstrate for the first time that alpha interferon (IFN-α) and ribavirin inhibit *in vitro* HEV replication in both a subgenomic replicon and an infectious culture system based on a genotype 3 strain. IFN-α showed a moderate but significant synergism with ribavirin. These findings corroborate the reported clinical effectiveness of both drugs. In addition, the antiviral activity of ribavirin against wild-type genotype 1, 2, and 3 strains was confirmed by immunofluorescence staining. Furthermore, the *in vitro* activity of ribavirin depends on depletion of intracellular GTP pools, which is evident from the facts that (i) other GTP-depleting agents (5-ethynyl-1-β-D-ribofuranosylimidazole-4-carboxamide [EICAR] and mycophenolic acid) inhibit viral replication, (ii) exogenously added guanosine reverses the antiviral effects, and (iii) a strong correlation (R^2=0.9998) exists between the antiviral activity and GTP depletion of ribavirin and other GTP-depleting agents.

Introduction

Hepatitis E virus (HEV) is a positive-sense, single-stranded RNA virus and is classified as the sole member of the Hepevirus genus in the Hepeviridae family [1,2]. HEV is usually transmitted feco-orally and may cause self-limiting acute hepatitis. At least four genotypes are currently recognized: genotypes 1 and 2 seem to infect only humans and are endemic in developing regions, with an estimated 70,000 deaths annually [3], while genotypes 3 and 4 are zoonotic agents, with domestic pigs as their main reservoir [1,2]. The latter two genotypes cause sporadic autochthonous infections in both developing and industrialized parts of the world, e.g., through consumption of undercooked pig meat [1,2]. In addition, related viruses have been found in several animal species, including chickens (avian HEV) [4], rats [5], rabbits [6], ferrets [7], bats [8], and trout (cutthroat trout virus [CTV]) [9].

Infections with HEV are often severe in pregnant women infected with genotype 1, with up to 25% mortality [1,10]. In addition, genotype 3 can cause chronic infections, particularly in immunocompromised individuals [11]. The first consideration in treating chronic hepatitis E, especially in transplant patients, is lowering immunosuppressive therapy, which induces clearance in over 30% of cases [12]. An additional course of pegylated alpha interferon (IFN-α) for 3 months or longer proved successful in most cases described [13], but ribavirin (RBV) monotherapy is probably the most frequently used option for chronic hepatitis E [11], and it also seems effective in severe acute infections [14,15].

Research on HEV has long been hampered by a lack of efficient cell culture models; however, *in vitro* cultures have been established in recent years [16,17]. By employing a replicon, an infectious virus yield assay, and immunofluorescence staining, we investigated the antiviral activities of both IFN-α and RBV against *in vitro* HEV replication. Depletion of intracellular GTP pools was found to be an important aspect of the mechanism of action of RBV.

Materials and Methods

***In vitro* transcription and capping.** HEV Kernow-C1 p6 (GenBank accession number JQ679013) and p6/luc-encoding RNAs were transcribed *in vitro* from MluI-digested plasmid DNA [17] by use of the T7 RiboMAX Large Scale RNA production system (Promega, Madison,

WI) and were capped with the ScriptCap m7G capping system (Cellscript, Madison, WI). To generate a transfection control for luminescence-based antiviral assays, the T7-IRES-FFLuc-YFsfRNA DNA fragment was PCR amplified from pT7-IRES-FFLuc-YFsfRNA with primers 5'-CATATGTCGACTAATACGACTCACTATAGGGATCCGCCCCTCTCCC-3' and 5'-AGTGG TTTTGTGTTTGTCATCC-3' and with Kapa HiFi HotStart ReadyMix master mix (Kapa Biosystems, Woburn, MA). The resulting fragment was *in vitro* transcribed. The internal ribosome entry site (IRES) alleviates the need for capping, and the yellow fever virus-derived small flaviviral RNA (YFsfRNA) enhances the cellular stability of this firefly luciferase (FFLuc)-encodingRNA [18]. The generation of pT7-IRES-FFLuc-YFsfRNA will be described elsewhere.

Cells and viruses.

Huh7 cells (a kind gift from Ralf Bartenschlager, University of Heidelberg, Heidelberg, Germany) and HepG2/C3A cells (a kind gift from Luc Verschaeve, Scientific Institute of Public Health, Brussels, Belgium) were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS; Integro, Leuvenheim, Netherlands) in a humidified 5% CO2 incubator at 37°C. To obtain infectious HEV stocks, HepG2/C3A cells seeded in a 6-well plate (BD Falcon, Franklin Lakes, NJ) were transfected with capped Kernow-C1 p6RNAby use of DMRIE-C (Invitrogen, Carlsbad, CA) and were incubated at 35°C. Part of the culture medium was changed every 2 to 3 days to ensure long-term cell survival, and culture medium was harvested from the original plate after 34 days of incubation. The obtained virus stock was propagated once more in HepG2/C3A cells, with weekly changing of half of the culture medium. Culture medium was harvested after 22 days. Since the Kernow-C1 p6 strain has been adapted to growth in HepG2/C3A cells [19], culturing of HEV p6 virus stocks in these cells resulted in higher-titer stocks than those obtained by culture in Huh7 cells (results not shown).

HEV strain Sar 55 (GenBank accession number M80581), Akluj (accession number AF107909), LBPR-0379 (accession number JN564006), and Kernow-C1 (accession number HQ389543) inocula were obtained from human stool samples, while the human Mex 14 strain (accession number M74506) was passaged once in a rhesus macaque. The work with human isolates was carried out under approved protocols of the Carolinas Medical Center (IRB-10-0709B) and the Royal Cornwall Hospital Trust (IRB-06Q2101/61), with the informed

consent of the patients. The housing, maintenance, and care of the rhesus macaque met or exceeded all requirements for primate husbandry as specified in the Guide for the Care and Use of Laboratory Animals [20].

Compounds.

IFN-α 2a (Roferon-A) was purchased from Roche Pharmaceuticals (Basel, Switzerland), diluted to 3×10^5 international units (IU)/ml in phosphate-buffered saline (PBS; Lonza, Basel, Switzerland) supplemented with 10% glycerol and 0.1% bovine serum albumin, stored at -80°C, and kept at 4°C after thawing. RBV [1-(β-D-ribofuranosyl)-1H-1,2,4-triazole-3-carboxamide (Virazole)] was purchased from ICN Pharmaceuticals (Costa Mesa, CA). 5-Ethynyl-1-β-D-ribofuranosylimidazole-4-carboxamide (EICAR) was a gift from Akira Matsuda (Hokkaido University), and mycophenolic acid (MPA) was purchased from Santa Cruz Biotechnology (Dallas, TX). Guanosine was from Sigma-Aldrich (St. Louis, MO). Stock solutions of the compounds were made in dimethyl sulfoxide (DMSO) and stored at 4°C.

Luminescence-based antiviral assay.

Huh7 cells were seeded in 96-well plates (BD Falcon) at 7.5×10^3 cells per well in 100 µL of DMEM with 10% FBS and were incubated at 37°C. After 24 h, cell layers were washed once with DMEM and transfected with IRES-FFLuc-YFsfRNA and capped p6/luc RNA (100 ng per well [each]) by use of DMRIE-C reagent (0.2 µL per well) according to the manufacturer's instructions. For cell control (CC) wells, viral RNA was omitted. Plates were incubated at 37°C for 4 h. Afterwards, the transfection medium was removed, cell layers were washed twice with PBS (100 µL per well), and 100 µL of compound diluted in DMEM with 10% FBS was added to each well. For virus control (VC) and CC wells, the compound was omitted. After incubation at 35°C for 3 days, 20 µL of the culture medium was transferred to a white 96-well CulturPlate (PerkinElmer, Waltham, MA), and luminescence produced by the secreted Gaussia luciferase (GLuc) was determined after addition of 50 µL of diluted Renilla luciferase assay substrate (Promega).Apilot study indicated that incubation for 3 days was sufficient to obtain a 100-fold increase in Gaussia-generated luminescence (data not shown), allowing for a fast and relatively sensitive readout. The remaining culture medium was removed, and 20 µL of passive lysis buffer (Promega) was added to each well. Next, the buffer with lysed cells was transferred to a white 96-well CulturPlate containing 100 µL of

diluted FFLuc assay substrate (Promega) per well, and luminescence counts were obtained. GLuc luminescence values were normalized with the following formula: $LucNorm_{compound}$ = ($GLuc_{replicon+compound}$-$Gluc_{CC}$)/$FFLuc_{replicon+compound}$. The percent antiviral activity was calculated as 100 - ($LucNorm_{compound}$/$LucNorm_{VC}$ × 100). The 50% effective concentration (EC50) was defined as the concentration of compound that caused a 50% reduction in the LucNorm signal compared to that of VC and was calculated through logarithmic interpolation. For toxicity evaluation, plates were prepared in parallel with antiviral assay mixtures, but the transfection step was omitted. After incubation for 3 days at 35°C, medium was removed and replaced with 100 µL of a 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium/phenazinemethosulfate (MTS/PMS; Promega) solution, as described previously [21]. After an incubation period of 2 h at 37°C, the optical density (OD) at 498 nm was determined for each well. The percent cell viability was calculated as follows:%viability=$OD_{compound}$/OD_{CC} × 100.

For combination studies, a checkerboard with 3-fold dilutions of RBV and 4-fold dilutions of IFN-α was used. Data were analyzed with the MacSynergy II template at the 95% confidence level [22]. Volumes of synergy or antagonism below 25 μM^2 % are considered insignificant, while values between 25 and 50 μM^2 % are minor but significant. Volumes between 50 and 100 μM^2 % indicate moderate synergy or antagonism [22–24].

Infectious virus yield assay

Huh7 cells were seeded in 6-well plates at 5 ×10^5 cells per well in 2 ml of DMEM with 10% FBS, penicillin (100 units/ml; Gibco), streptomycin (100 g/ml; Gibco), and amphotericin B (Fungizone) (2.5 g/ml; Gibco) and were incubated at 37°C. After 24 h, culture medium was removed and cell layers were inoculated with infectious p6 virus at 2.1 × 10^5 RNA copies/ml in 1ml of medium with DMSO (0.5%) or compound and then incubated at 35°C. After 5 h, the inoculum was removed, cell layers were washed 3 times with 2ml of PBS, and 3ml of medium with DMSO or compound was added to each well. After 1 h, a 150 µL sample was taken and stored at -80°C. Since HEV replication *in vitro* is rather poor, regular changing of the culture medium was required to allow cell survival over the full duration of the assay. Consequently, 750 µL was removed from each well every 2 to 3 days and stored at -80°C, and 1 ml of fresh medium with DMSO or compound was added. The viral loads in samples

from 1 h, 12 or 13 days, and 20 days post infection were determined by reverse transcription-quantitative PCR (RT-qPCR). Statistical significance was calculated with the Mann-Whitney U test. On day 20 postinfection, all medium was removed from the plate, cell layers were washed with 2 ml of PBS, and 1ml of MTS/PMS solution was added for evaluation of compound cytotoxicity. Cultures were incubated for 2 h at 37°C, and subsequently, the OD at 498 nm was determined for 100 µL from each well. Cell viability was expressed as a percentage of the DMSO control level.

Immunofluorescence analysis and focus-forming assay

HEV strains Sar 55, Akluj, Mex 14, LBPR-0379, Kernow-C1, and Kernow-C1 p6 were inoculated onto HepG2/C3A cells seeded in 8-well Lab-Tek II CC2 chamber slides (Nunc, Penfield, NY). Cells in triplicate wells were incubated with medium with or without RBV at 200 µM. After 3 days, cells were fixed and stained for capsid protein as described before [19]. Each well was manually scanned, and fluorescent cells or foci were counted. Cell viability of HepG2/C3Acells treated with RBV at 200 µM for 3 days was assessed with the MTS/PMS method. RBV was used at a higher concentration than that in Huh7 cells because HepG2 cells appear to require higher concentrations to induce intracellular GTP depletion (e.g., see reference [25]).

Quantification of intracellular GTP concentrations

RBV, MPA, or EICAR was added to 1-day-old cultures of Huh7 cells in DMEM with 10% FBS in 25-cm^2 flasks. For RBV, concentrations of 2.5, 10, 25, and 100 µM were tested. For MPA and EICAR, concentrations of 0.1, 0.25, 1, and 2.5 µM were used. Untreated CC cultures were included as well. Flasks were incubated at 35°C for 3 days, and subsequently, cells were detached using trypsin-EDTA solution (Gibco) and collected by centrifugation. Nucleotides were extracted from the cell pellet, and GTP levels were quantified by high-performance liquid chromatography as described previously [26]. The 50% inhibitory concentration (IC50) was defined as the concentration of compound required to reduce the GTP levels to 50% of the levels in untreated control cultures.

RT-qPCR.

Viral RNA was extracted from culture medium by use of a NucleoSpin RNA virus kit (Macherey-Nagel, Düren, Germany) according to the manufacturer's instructions. Primer and probe sequences for TaqMan-based quantification of HEV RNA were published previously [27]. The forward primer 5'-GGTGGTTTCTGGGGTGAC-3' and the reverse primer 5'-AGGGGTTGGTTGGATGAA-3' were used. The probe was labeled with 6-carboxyfluorescein (FAM) at the 5' end and with a minor groove binder (MGB) at the 3' end (5'-FAM-TGATTCTCAGCCCTTCGC-MGBNFQ-3') [28]. Reactions were performed with One-Step qRT-PCR mix (Eurogentec, Seraing, Belgium) in a final volume of 25 µL containing 250 nM of each primer, 100 nM of probe, and 5 µL of RNA sample. PCR was performed using an ABI 7500 Fast real-time PCR system (Applied Biosystems, Foster City, CA) under the following conditions: 30 min at 48°C and 10 min at 95°C, followed by 40 cycles of 15 s at 95°C and 1 min at 60°C. Data were analyzed with ABI Prism 7500 SDS software (version 1.3.1; Applied Biosystems). For absolute quantification, standard curves were generated using 10-fold dilutions of the cloned target cDNA.

Results

We used a transient GLuc-expressing genotype 3 replicon (Kernow-C1 p6/luc) [17] in Huh7 hepatoma cells to assess the potential anti-HEV activity of RBV and IFN-α, two drugs whose clinical efficacy has been reported in several case studies of HEV-infected patients [11,13]. Both RBV and IFN-α efficiently inhibited HEV replication, with EC50s of 3 ± 2 µM (0.7 ± 0.5 µM/ml) and 1.3 ± 0.5 IU/ml, respectively (Figure 1A and B). The combination of both inhibitors resulted in a moderately synergistic antiviral effect, with a synergy volume of 72 μM^2 % (Figure 1C). The combined antiviral activity was maximally 22% above the expected value (at about 4 IU/ml IFN-α and 0.4 M RBV).

Next, the potential antiviral activity was assessed in Huh7 cells infected with the Kernow-C1 p6 virus [17]. Viral replication was quantified by means of RT-qPCR detection of viral RNA in the culture medium. Both IFN-α and RBV resulted in significant reductions of viral titers at 12 and 20 days postinfection (Figure 1D), without decreasing cell viability (RBV concentrations of up to 100 µM for 20 days) (Figure 1F). A dose-dependent inhibition of viral replication was observed with RBV, with a 4.1-$\log_{10}$ reduction in viral titer after 20 days at a

concentration of 100 µM. Some differences in the antiviral potency of RBV observed between the replicon and virus yield assays, though limited, may be explained by the different setups (luminescence versus quantification of released viral RNA) and the respective time windows (3 versus 20 days) of the assays.

Since RBV was reported to be effective in the treatment of acute genotype 1 infections [14,15], the antiviral activity of RBV against genotypes 1 and 2 was assessed. To this end, HepG2/C3A cells were infected with wild-type isolate Sar 55, Akluj (both genotype 1), or Mex 14 (genotype 2) and stained for capsid protein. These strains do not spread in cell culture but are able to replicate intracellularly. RBV treatment (200 µM) reduced the number of fluorescent foci to almost zero (Figure 1E). Comparable results were obtained for the wild-type genotype 3 strains LBPR-0379 and Kernow-C1 and for the cell culture-adapted Kernow-C1 p6 strain. No cytotoxicity was observed for RBV at 200 µM for 3 days (Figure 1G).

In addition, treatment of Sar 55 Gluc-containing replicon cells with RBV resulted in a pronounced reduction of viral replication, even when addition of RBV was delayed until 4 days posttransfection and activity was assessed at 15 days posttransfection (data not shown).

One of the proposed mechanisms of action for the broad-spectrum antiviral activity of RBV is that inhibition of IMP dehydrogenase (IMPDH) by RBV 5'-monophosphate results in depletion of the intracellular GTP pools, thus impeding RNA virus replication [29,30]. To determine whether GTP depletion is involved in inhibition of *in vitro* HEV replication, we assessed the antiviral activities of two known IMPDH inhibitors: MPA (an immunosuppressive agent) and EICAR, a 5-ethynylimidazole analogue of RBV [31,32]. Marked inhibition of HEV replication was observed in the replicon assay, with EC50s of 0.20 ± 0.04 µM and 0.115 ± 0.007 µM for MPA and EICAR, respectively (Figure 2A and B).

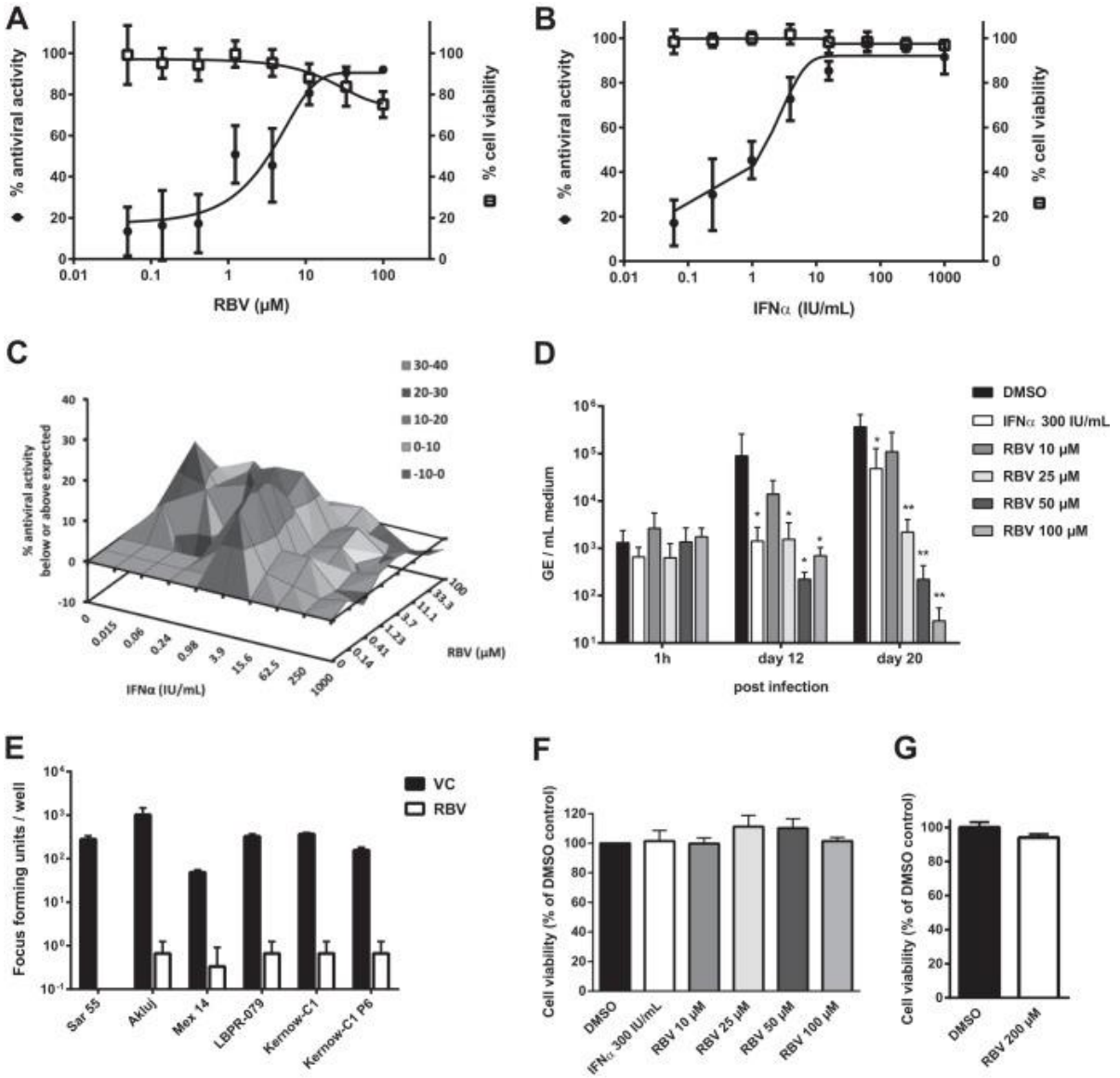

Figure 1. Inhibitory activities of IFN-α and RBV against HEV replication. Huh7 cells were transfected with capped p6/luc RNA and treated with RBV (A) or IFN- α(B) for 3 days. (C) Antiviral activities below or above those expected for RBV–IFN-α combinations. (D) Antiviral activities were also assessed in an infectious virus yield assay with RT-qPCR detection of viral RNA. *, $P < 0.05$; **, $P < 0.01$. GE, genome equivalents. (E) RBV (200 μM) inhibited formation of foci in HepG2/C3A cells infected with strains Sar 55, Akluj, Mex 14, LBPR-0379, Kernow-C1, and Kernow-C1 p6. (F) Cell viability of Huh7 cells used in the infectious virus yield assay after 20 days in the presence of IFN-α or ribavirin, as assessed by the MTS/PMS method. (G) Cell viability of HepG2/C3A cells treated with RBV at 200 μM for 3 days was assessed by the same method. Values represent means standard deviations (SD) for at least 3 independent experiments (A to D, F, and G) or 3 replicates (E).

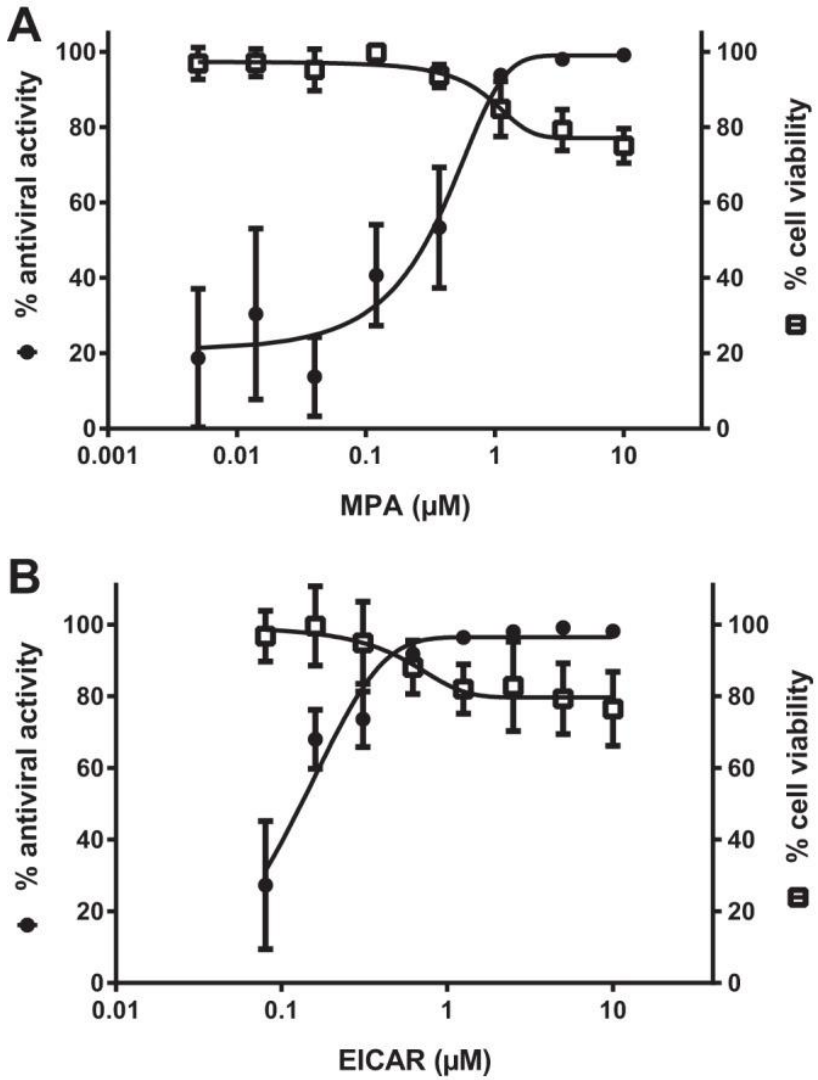

Figure 2. Dependence of the antiviral activity of RBV on the depletion of intracellular GTP pools. MPA (A) and EICAR (B) are known inhibitors of IMPDH and are potent inhibitors of HEV replication in the replicon assay. Values represent means SD for at least 3 independent experiments.

It has been shown for several viruses that are sensitive to RBV that replenishing of intracellular GTP pools through addition of exogenous guanosine restores virus replication [29,33]. Concordantly, the anti-HEV activities of RBV, MPA, and EICAR were reversed following addition of guanosine to the cell culture medium (Figure 3A to C). Next, we quantified the intracellular GTP pools of Huh7 cells and the effects of different concentrations of the three compounds. Normal GTP concentrations were calculated to be around 350M, and RBV, MPA, and EICAR effectively depleted GTP pools, with IC50s of 18 ± 9 µM, 0.5 ± 0.1 µM, and 0.3 ± 0.2 µM, respectively. A strong correlation between both the IC50s for GTP depletion and the respective antiviral EC50s was calculated (R^2 = 0.9998) (Figure 3D).

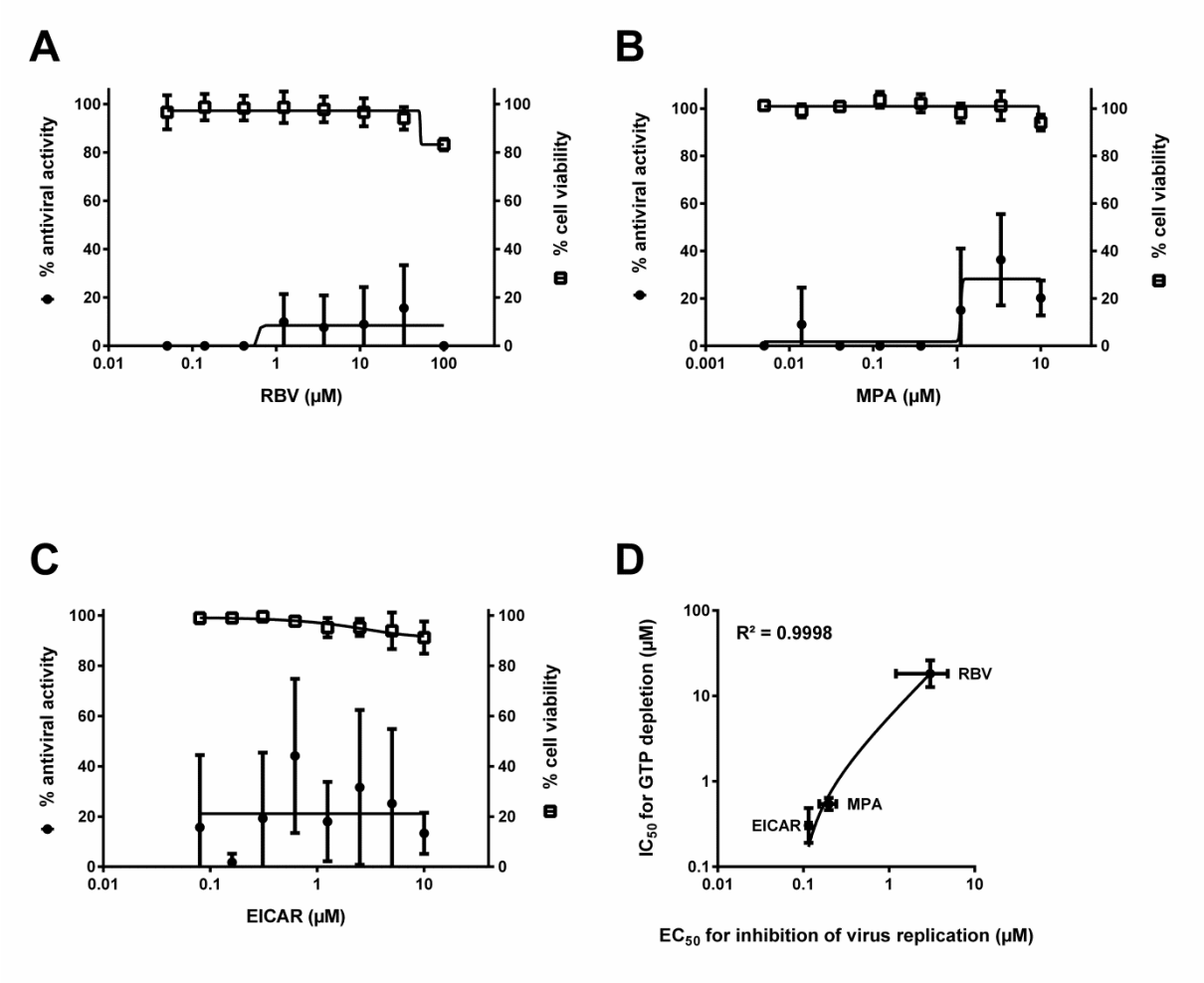

Figure 3. Replenishing GTP pools salvages HEV replication. Addition of exogenous guanosine to the culture medium (40 μM) salvages HEV replication and abolishes the antiviral effects of RBV (A), MPA (B), and EICAR (C) in the replicon assay. (D) Relationship between the IC50s for GTP depletion and EC50s for inhibition of viral replication. Values represent means SD for at least 3 independent experiments.

Discussion

Pegylated IFN-α and RBV are the only drugs that are currently available to treat acute and chronic hepatitis E [5,7,9]. Treatment duration is at least 12 weeks for chronic infections [13] and a minimum of 3 weeks for acute infections [14,15,34] (compare with the current standard of care for hepatitis C, i.e., pegylated IFN-α plus RBV for up to 48 weeks, combined with a protease inhibitor for genotype 1 infections [35]). The scientific evidence for these hepatitis E treatments is limited to a number of case series and the *in vitro* activity against CTV, a HEV surrogate [36]. None of these interventions or the superiority of one over the other has been validated in controlled trials yet. Here we demonstrate the antiviral activity of IFN-α and RBV against HEV *in vitro*, thus providing additional evidence for the clinical use of these drugs. In addition, a moderate but statistically significant synergy was calculated for the

combination of IFN-α and RBV (Figure 1C). The clinical relevance of this observation is unclear, however, since antiviral activity was maximally 22% above that expected. Nevertheless, since no antagonistic effects were observed, it might be an option to treat patients with a combination of IFN-α and RBV, as is the case in the management of infections with hepatitis C virus (HCV). Lower doses of both drugs may possibly be sufficient in a combination regimen, thus reducing the frequency and severity of adverse effects. In fact, successful combination therapy has already been reported for a chronically HEV-infected HIV patient [37].

When the *in vitro* inhibitory concentrations of IFN-α and RBV are compared to the serum concentrations typically obtained in (HCV-infected) patients, they seem to be in a roughly similar concentration range. For instance, mean pegylated IFN-α serum concentrations of around 80 IU/ml have been reported [38], which is considerably higher than the EC50 for inhibition of the HEV replicon, although this is lower than the 300 IU/ml that resulted in a 0.9-$\log_{10}$ reduction in the virus yield assay. For RBV, serum concentrations between 8 and 13 µM have been reported [38,39], and hepatic accumulation of ribavirin has been claimed, with a steady-state liver concentration of 250 µM [40]. We calculated an EC50 of 3 µM for RBV and observed strong antiviral effects in the virus yield assays for concentrations above 10 µM. Although the relevance of such comparisons may be questionable, the fact that both IFN-α and RBV are active *in vitro* is in agreement with the observed clinical efficacy of both in chronically HEV infected patients.

In a recent study by Dong and colleagues, the HEV ORF3 protein was shown to inhibit IFN-α signaling [41]. In line with this finding, high concentrations of IFN-α were required to reduce release of HEV RNA into the culture medium after infection (e.g., 50% reduction at concentrations as high as 1,000 units/ml). A similarly high concentration of 300 IU/ml was needed in our infectious HEV system to achieve reductions of viral RNA levels in the virus yield assay of 98% and 87% at 12 and 20 days postinfection, respectively (Figure 1D). In the replicon assay, on the other hand, we observed strong inhibition by IFN-α at concentrations as low as 10 IU/ml (Figure 1B). Slight differences obtained in the infectious HEV system may be explained by the use of different cell lines (A549 versus Huh7) and virus isolates and/or the fact that the HEV infection was already established before the start of IFN-α treatment in the study of Dong et al., while IFN-α was included in the inoculum during initial infection

in our virus yield system. However, the pronounced potency of IFN-α against the subgenomic HEV replicon system can readily be explained by the lack of an interferon-antagonizing activity in p6/luc, where expression of ORF3 is ablated by replacement with a reporter cassette, making the replicon especially susceptible to inhibition by IFN-α. This is fully in line with the findings of Dong et al. [41].

The data presented here indicate that depletion of cellular GTP pools is the predominant mechanism by which ribavirin inhibits *in vitro* HEV replication. It is unclear, however, to what extent hepatic GTP levels can be decreased by RBV *in vivo* [30]. Other proposed mechanisms of action of RBV include immunomodulatory effects, modulation of IFN-α stimulated gene expression, lethal mutagenesis forcing the virus into an error catastrophe, interference with viral methyltransferase activity, direct inhibition of the viral polymerase, and inhibition of eukaryotic initiation factor 4E interfering with cap-dependent translation [30]. Although one or several of these mechanisms could be involved in the antiviral activity of RBV against HEV, the results presented here suggest that GTP depletion is an important mechanism contributing to its antiviral activity.

Interestingly, the use of mycophenolate mofetil, a prodrug of MPA, for immunosuppressive therapy in transplant patients with chronic hepatitis E was significantly associated with HEV clearance [42]. This observation is in accordance with the strong antiviral activity observed in our replicon assay (Figure 2A). However, it is questionable whether the antiviral effect outweighs the immunosuppression that initially allowed HEV to establish a chronic infection.

The replicon-based antiviral assay as described here is rather labor-intensive, but to our knowledge, it is the first reported system that allows testing of potential antiviral molecules targeting HEV replication. In addition, the results of the infectious virus yield indicate that it is feasible to study the impact of potential antiviral compounds on the replication of full-length replication competent HEV. Although the isolation of the Kernow-C1 p6 strain was a major step forward, the replication kinetics are (still) rather slow, thus requiring long incubation times to obtain sufficiently high HEV titers for detection by RT-qPCR. Further adaptation to cell culture may be a possible solution; this strategy proved

successful, for instance, for hepatitis A virus and allowed for the development of relatively fast virus yield assays [43].

In conclusion, the observed *in vitro* antiviral activities provide additional support for the clinical use of both RBV and (pegylated) IFN-α for the treatment of severe cases of hepatitis E. However, both therapies require long treatment periods and can have severe adverse effects. RBV dose reductions because of anemia have resulted in treatment failure and death in some cases [15]. Consequently, the assays presented here may serve as a starting point for developing convenient systems for high-throughput screening. This should allow the development of more potent anti-HEV drugs with a better safety profile. Ideally, these drugs should be safe during pregnancy. Since HEV appears to be emerging and its impact on public health has been underestimated, it would be wise to invest in research toward such antivirals.

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CHAPTER 6

Crosstalk between Nucleotide Synthesis Pathways with Cellular Immunity in Constraining Hepatitis E Virus Replication

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Abstract

Viruses are solely dependent on host cells to propagate, therefore understanding virus-host interaction is important for antiviral drug development. Since *de novo* nucleotide biosynthesis is essentially required for both host cell metabolism and viral replication, specific catalytic enzymes of these pathways have been explored as potential antiviral targets. In this study, we investigated the role of different enzymatic cascades of nucleotides biosynthesis in hepatitis E virus (HEV) replication. By profiling various pharmacological inhibitors of nucleotides biosynthesis, we found that targeting the early steps of the purine biosynthesis pathway led to enhancement of HEV replication; whereas targeting the later step resulted in potent antiviral activity via depletion of purine nucleotide. Furthermore, inhibition of pyrimidine pathway resulted in potent anti-HEV activity. Interestingly, all these inhibitors with anti-HEV activity concurrently triggered the induction of antiviral interferon-stimulated genes (ISGs). Although ISGs are commonly induced by interferons via the JAK-STAT pathway, their induction by nucleotides synthesis inhibitors is completely independent of this classical mechanism. In conclusion, this study revealed an unconventional novel mechanism as to a crosstalk between nucleotide biosynthesis pathways and cellular antiviral immunity in constraining HEV infection. Targeting particular enzymes in nucleotide biosynthesis represents a viable option for antiviral drug development against HEV.

Author Summary

HEV is the most common cause of acute viral hepatitis worldwide and is also associated with chronic hepatitis, especially in immunocompromised patients. Although often an acute and self-limiting infection in the general population, HEV can cause severe morbidity and mortality in certain patients, a problem compounded by the lack of FDA-approved anti-HEV medication available. In this study, we have investigated the role of nucleotide synthesis pathway in HEV infection and its potential for antiviral drug development. We show that targeting the later but not the early steps of purine synthesis pathway exert strong anti-HEV activity. In particular, IMPDH is the most important anti-HEV target of this cascade. Importantly, the clinically used IMPDH inhibitors, including mycophenolic acid and ribavirin, have potent anti-HEV activity. Furthermore, targeting pyrimidine synthesis pathway also exerts potent antiviral activity against HEV. Interestingly, antiviral effects of nucleotide synthesis pathway inhibitors appear to depend on medication-induced transcription of antiviral interferon-stimulated genes. Thus, this study reveals an unconventional novel mechanism as to how nucleotide synthesis pathway inhibitors can counteract HEV replication.

Introduction

Hepatitis E virus (HEV) is a single-stranded positive-sense RNA virus, which mainly infects the liver. It is the most common cause of acute viral hepatitis worldwide. In general, HEV infection is a self-limiting disease and associated with low mortality, but epidemics of hepatitis E occur periodically throughout the developing world, resulting in 70,000 death yearly [1]. In western countries, HEV primarily affects immunocompromised patients, in particular organ transplant recipients, as well as hematopoietic stem cell transplant [2-5]. More than 60% of organ recipients infected with HEV develop chronic hepatitis with rapid progression to cirrhosis [2]. Despite an emerging global health issue, no FDA-approved anti-HEV therapy is currently available, only interferon-α, ribavirin or a combination have been occasionally used as off-label treatment. Thus, further research aimed at understanding its infection biology and developing effective antiviral treatment is urgently required.

Cellular nucleotides, including purines and pyrimidines, are the basic building blocks that form the nucleic acids RNA and DNA. Nucleotides are the fundamental components that are required for cell metabolism, such as genome replication. *In vivo*, nucleotides can be synthesized *de novo* through a series of enzymatic reactions or recycled through salvage pathways. Since viral replication heavily relies on the host cells to supply nucleosides, targeting nucleotide biosynthesis pathway thus represents an attractive strategy for antiviral drug development. The nucleotide biosynthesis pathways have been well-studied for decades [6-8]. Numerous compounds have been developed and well-characterized to target particular enzymes of this pathway to inhibit viral infections by depletion or causing imbalance of nucleotide pools [9-18]. Among them, inhibitors of inosine monophosphate dehydrogenase (IMPDH), a key enzyme of the purine synthesis pathway, have been successfully used in the clinic for decades. These drugs including ribavirin and mycophenolic acid (MPA), used as antiviral or immunosuppressive medication respectively, have been demonstrated to have broad antiviral activity against a spectrum of viruses, including dengue virus, yellow fever virus (YFV), hepatitis B, hepatitis C and hepatitis E virus [14,15,18-21]. Likewise, Brequinar and Leflunomide, the inhibitors of dihydroorotate dehydrogenase (DHODH), an essential enzyme of pyrimidine nucleotide synthesis, have been shown to inhibit human polyomavirus type BK (BKV), YFV and dengue virus [12,22].

Besides their function as building blocks of genetic material, free nucleotides also play important roles in cell signalling. We and others have previously reported the potential interaction of nucleotide deprivation and cellular antiviral immune response, such as provoking the expression of interferon-stimulated genes (ISGs) [19,23]. Given that the liver is a major site for nucleotide synthesis, we comprehensively profiled the role of purine and pyrimidine synthesis pathways in HEV cell culture models, aimed at identifying potential antiviral drug targets and understanding the crosstalk with cellular antiviral immunity against HEV infection.

Materials and Methods

Reagents

Guanosine (CAS: 118-00-3), Adenosine (CAS: 58-61-7), Uridine (CAS: 58-96-8), 6-TG (CAS: 154-42-7), Lometrexol hydrate (CAS: 106400-81-1), MTX hydrate (CAS: 133073-73-1), FA phosphate (CAS: 75607-67-9), BQR sodium salt hydrate (MDL: MFCD21363375), LFM (CAS: 75706-12-6) and 6-AU (CAS: 461-89-2) were purchased from sigma. 23 IMPDH specific inhibitors were kindly provided by Center for Drug Design, University of Minnesota. All the reagents were dissolved in dimethylsulfoxide (DMSO). The effects of these *de novo* nucleotide biosynthesis inhibitors on host cell viability were determined by MTT assay (Supplementary Figure 7). Stocks of JAK inhibitor 1 (CAS 457081-03-7, Santa Cruz Biotech, CA) was dissolved in DMSO with a final concentration of 5 mg/mL. Stocks of CP-690550 (Tofacitinib) (Santa Cruz Biotech, CA) were dissolved in DMSO with a final concentration of 10 mg/mL.

Cell culture

Human hepatoma cell line Huh7 and human embryonic kidney epithelial cell line 293T cells were cultured in Dulbecco's modified Eagle medium (DMEM) (Invitrogen) supplemented with 10% fetal bovine serum, 100IU/mL penicillin and 100IU/mL streptomycin.

Cell culture models

HEV replication model with subgenomic HEV sequence coupled with a Gaussia luciferase reporter gene and HEV infection model containing the full-length HEV genome were used in our study. The construction of two models has been described previously [18]. Besides, Huh7 cells constitutively expressing the firefly luciferase reporter gene driven by the human PGK promoter were used as household luciferase activity for normalizing nonspecific effects on luciferase Activity [11]. Huh7 cells transduced with lentiviral transcriptional reporter system expressing the firefly luciferase gene under control of a promoter containing multiple ISRE promoter elements (SBI Systems Biosciences, Mountain View, CA) was established and luciferase activity represents ISRE promoter activation.

Quantification of HEV replication and infection

The details for exanimating HEV replication and infection were described before [18]. Briefly, For the HEV replication model (p6-Luc), the activity of secreted gaussia luciferase in the cell culture medium was measured using BioLux® *Gaussia* Luciferase Flex Assay Kit (New England Biolabs), as quantification of viral replication, which was normalized by firefly luciferase expression. For full- length HEV infectious model, SYBR Green based qRT-PCR was used to quantify the newly formed viral genomic RNA after cell lysis and the HEV primer sequences were shown in supplementary Table 2.

Gene knockdown by lentiviral vector delivered short hairpin RNA (shRNA)

Lentiviral vectors, targeting PPAT, GART, ATIC, DHODH, were produced in 293T cells as previously described [11]. To generate stable gene knockdown cells, Huh7 cells were transduced with lentiviral vectors. Since the vectors also express a puromycin resistance gene, transduced cells were subsequently selected by adding 2.5 μg/ml puromycin (Sigma) in the cell culture medium. After pilot study, the shRNA vectors (Supplementary Figure 1 and Supplementary Table 3) exerting optimal gene knockdown were selected by qPCR with the corresponding primers shown in supplementary Table 2. Meanwhile, shRNA vector expressing Green fluorescent protein (GFP) was used as control (shCTR). The amount of HEV were assessed after 3 days of infectious HEV medium post-infecting shGFP cells and knockdown cells. For the experiment comparing the activity of compounds between shGFP

and knockdown cells, infectious HEV cells were directly transduced with lentiviral shRNA vectors and selected by puromycin.

Statistical analysis

Statistical analysis was performed using the nonpaired, nonparametric test (Mann–Whitney test; GraphPad Prism software). P values less than 0.05 were considered as statistically significant.

Results

Exogenous guanosine, but not uridine, stimulates HEV replication

Purine and pyrimidine nucleotides are the major cellular energy carriers and constitute the defining subunits of nucleic acids. Two distinct pathways are responsible for the biosynthesis of these two types of nucleotides (Figure 1A and 2A). Their fundamental role in cellular biochemistry raises the possibility that modifying flux through nucleotide biosynthesis pathways would profoundly influence the course of viral infection. Thus we decided to assess the overall impact of either purine or pyrimidine synthesis on HEV infection. A first indication that such effects might exist came from experiments in which we arbitrarily increased the purine and pyrimidine content by supplementation of exogenous guanosine (Figure 1A) and uridine (Figure 2A) in human hepatoma cell line (Huh7)-based HEV cell culture models. Guanosine, a purine nucleoside containing guanine attached to a ribose, can be converted to guanosine monophosphate (GMP) through purine salvage synthesis pathway, subsequently replenishes purine nucleotide pool (Figure 1A). Mechanistically, the cleavage of exogenous guanosine was catalysed by purine nucleoside phosphorylase (PNP) to form guanine. In the presence of hypoxanthine/guanine phosphoribosyl transferase (HGPRT), guanine was converted to GMP by addition of ribose 5-phosphate from phosphoribosyl pyrophosphate (PRPP). Supplementation of guanosine dose-dependently enhanced HEV replication-related luciferase activity in the subgenomic replicon (p6-Luc) model and increased cellular viral RNA in the full-length (p6) infectious model (Figure 1B). Likewise, uridine, which is a pyrimidine nucleoside consisting of uracil binding to ribose, commonly presents as uridine monophosphate (UMP) to rescue cells from pyrimidine nucleotide depletion (Figure 2A). In contrast, supplementation of exogenous

uridine had no effect on HEV replication (Figure 2B). Thus interaction between at least some of the pathways involved in nucleotide biosynthesis and the HEV infectious process might exist.

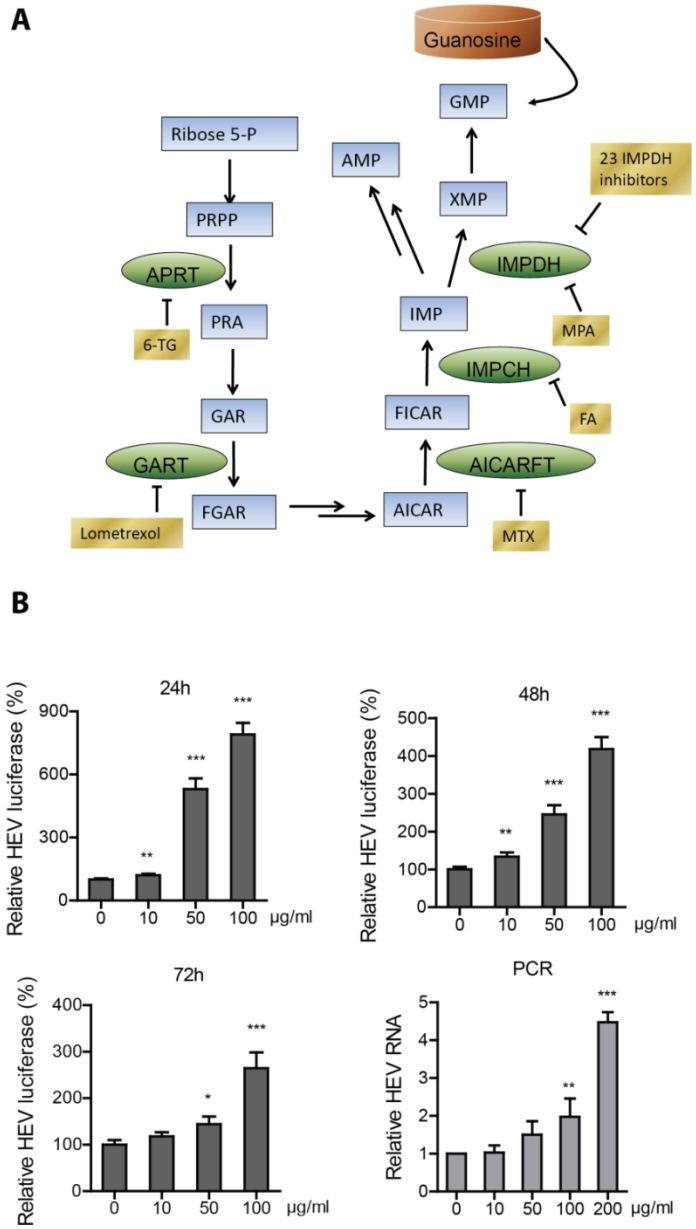

Figure 1. Exogenous guanosine stimulated HEV replication. (A) Schematic overview of *de novo* biosynthesis of purine nucleotide. PRPP, 5-phosphoribosyl-1-pyrophosphate; PRA, 5-phosphoribosylamine; GAR, glycinamide ribonucleotide; FGAR, formyl-GAR; AICAR, 5-aminoimidazole-4-carboxamide ribonucleotide. (B) Huh7 cell-based subgenomic HEV replicons containing the luciferase reporter gene were treated for 24 h, 48 h, and 72 h with a dose range of guanosine (n = 4). Data are presented as means standard errors of the means (SEM). Meanwhile, Huh7 cells with the infectious HEV containing the full-length p6 genome were treated for 48 h with a dose range of guanosine (n = 5). Data were normalized to two housekeeping genes (GAPDH and RP2) and are presented relative to the control (CTR) (set as 1). Data represent means ± SEM. *, $P < 0.05$; **, $P < 0.01$; ***, $P< 0.001$.

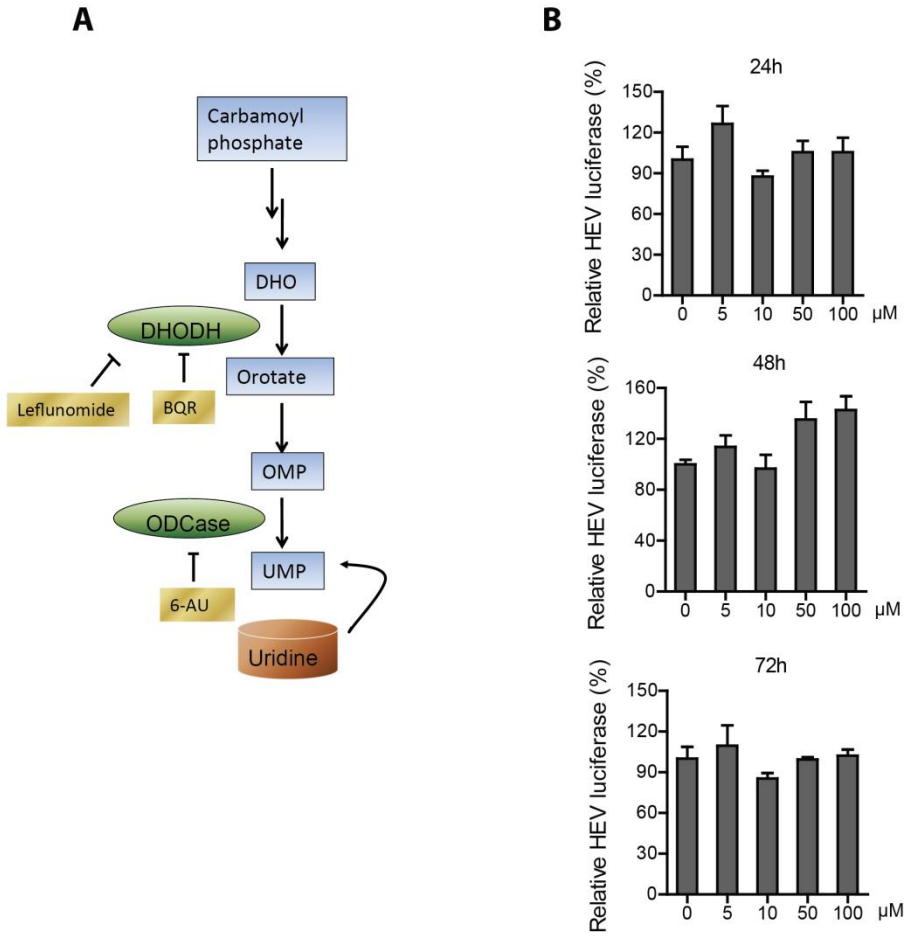

Figure 2. Exogenous uridine does not affect HEV replication. (A) Schematic overview of *de novo* biosynthesis of pyrimidine nucleotide. (B) Huh7 cell based subgenomic HEV replicon containing the luciferase reporter gene was treated for 24 h, 48 h, and 72 h with a dose range of uridine (n = 5). Data are presented as means ± SEM.

Targeting the catalytic steps leading to the primary purine nucleotide synthesis (inosine monophosphate ; IMP), stimulates HEV replication

Given the clear pro-viral effect of exogenous guanosine, we were encouraged to explore potential anti-HEV strategies targeting the different enzymes that are involved in purine nucleotide synthesis. *De novo* purine is mainly synthesized in the liver, which begins with the starting material 5-phosphoribosyl-1-pyrophosphate, PRPP. The first fully-formed nucleotide IMP is catalyzed through ten reactions by six enzymes (Figure 1A). We first selectively targeted three key enzymes of this cascade, including amido phosphoribosyltransferase (APRTase), glycinamide ribonucleotide trasnformylase (GART) and 5-aminoimidazole-4-carboxamide ribonucleotide formyltransferase (AICARFT) through 6-thioguanine (6-TG), lometrexol and methotrexate (MTX), respectively. Somewhat counterintuitively, all three compounds increased HEV replication in both cell culture

models (Figure 3). To further clarify the role of their targets, lentiviral-mediated RNAi was used for knockdown of these three genes PPAT, GART and ATIC that encode the corresponding enzymes APRTase, GART and AICARTF, respectively (Figure 4A). Consistent with the pharmacological results, down-regulation of these enzymes enhanced HEV replication (Figure 4B). Furthermore, the pro-viral effects of the pharmacological inhibitors were largely absent in a context in which their targets were silenced, suggesting that pharmacological effects are not due to off-target effects (Figure 4C).

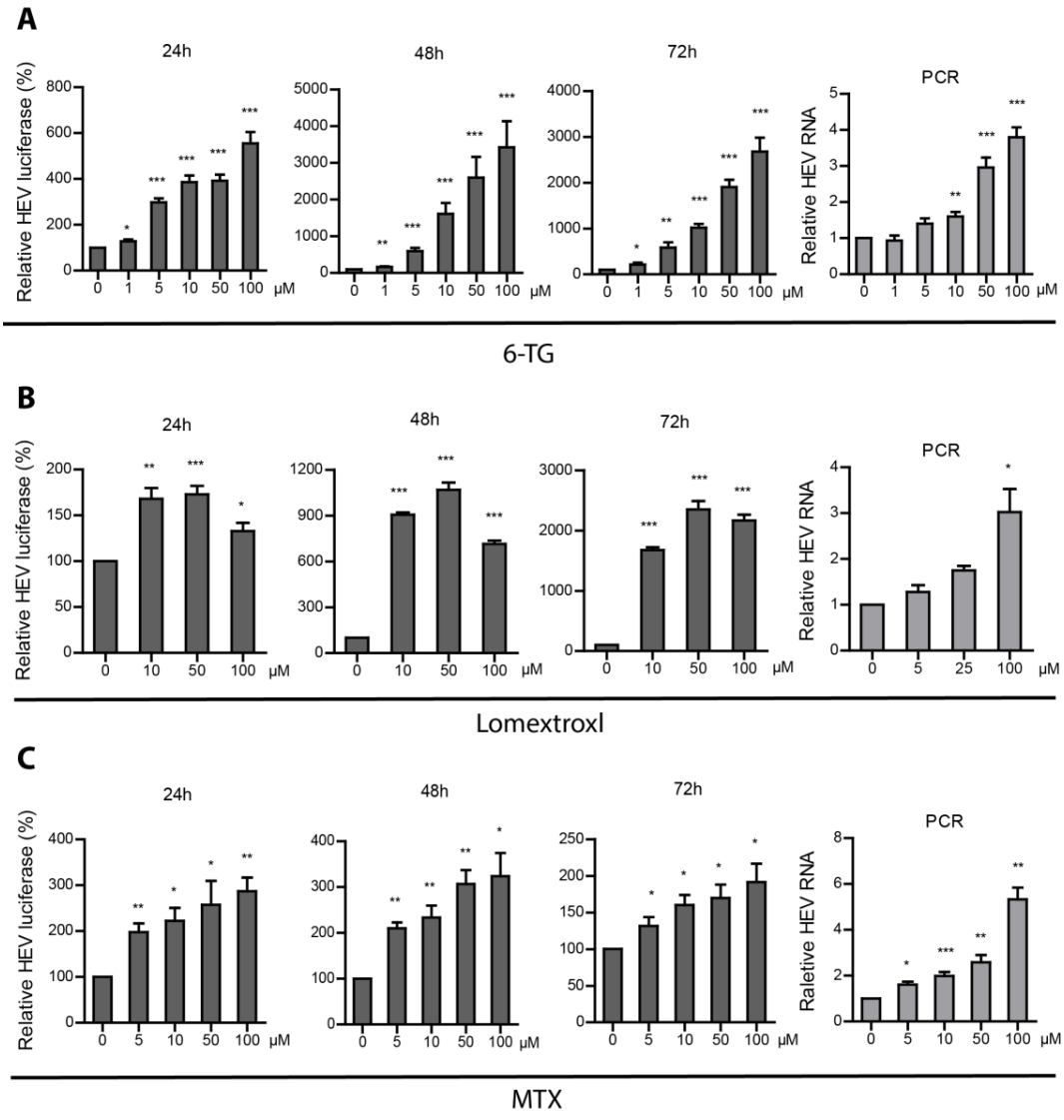

Figure 3. Inhibitors of IMP synthesis cascade stimulate HEV replication. The Huh7 cells containing subgenomic HEV replicons with luciferase reporter genes were incubated with increasing doses of 6-TG (A), lometrexol (B), and MTX(C). The luciferase activity was determined at 24 h, 48 h, and 72h. Accordingly,Huh7 cells infected with full-length HEV were treated with increasing doses of 6-TG (A), lometrexol (B), and MTX (C). The HEV RNA level was quantified by qRT-PCR after 48 h. Data were normalized to two housekeeping genes and are presented relative to the control (CTR) (set as 1). Data represent means ± SEM from five to eight experiments. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

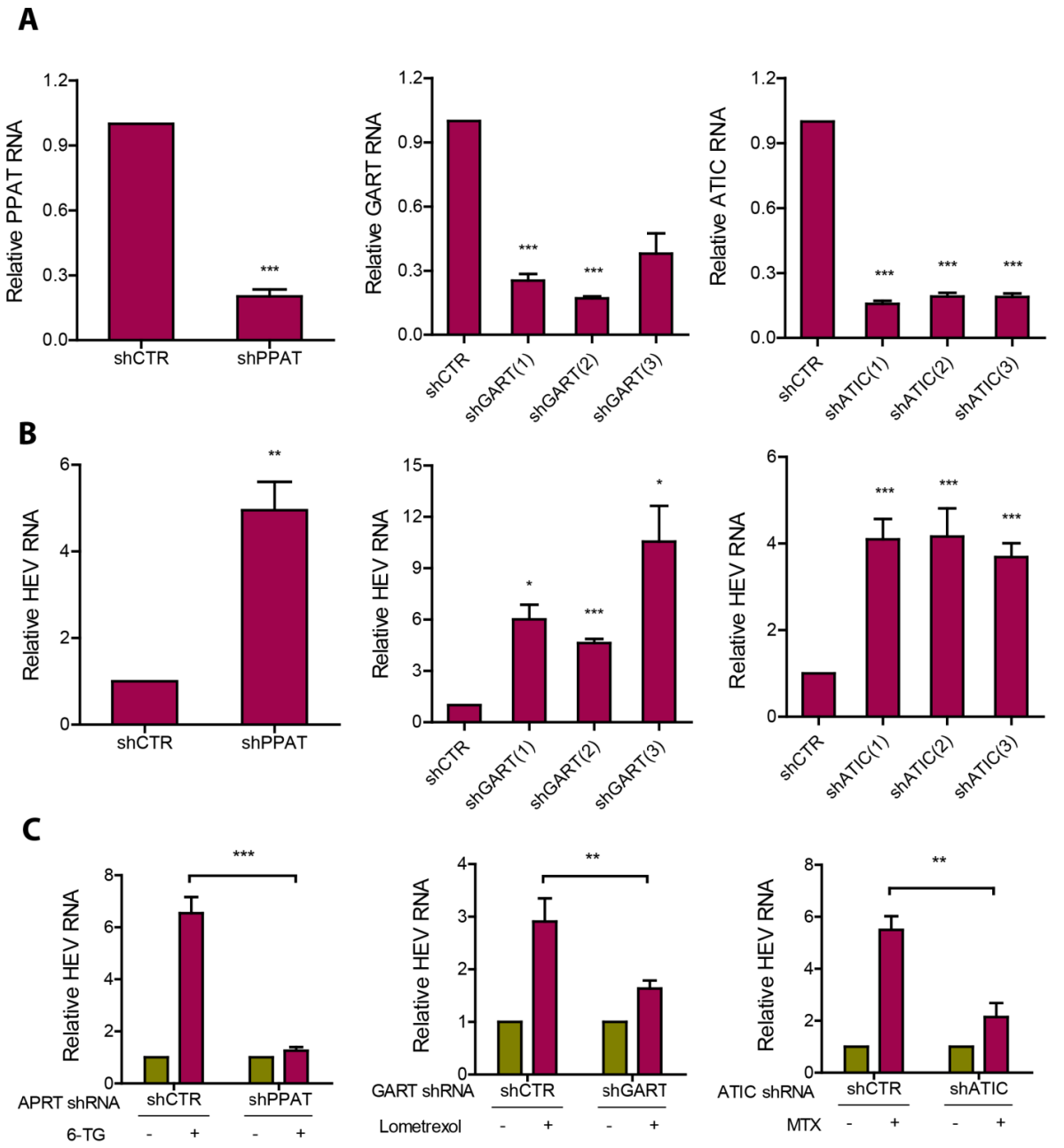

Fig 4. Silencing of enzymes involved in IMP synthesis cascade facilitates HEV replication. (A) Huh7 cells were transduced with lentiviral shRNAs to stably silence the corresponding genes for PPAT, GART, and ATIC (a set of independent shRNA clones targeting each gene was used). Huh7 cells transduced with lentiviral shRNA targeting GFP (shCTR) were used as a control. The efficiency of gene knockdown was analyzed by qRT-PCR. (B) Silencing of PPAT, GART, and ATIC resulted in significant elevation of viral RNA upon inoculation of HEV. HEV RNA levels were determined 72 h after inoculation. (C) Silencing of PPAT, GART, and ATIC abrogated the pro-HEV effects of 6-TG, lometrexol, and MTX. Data were normalized to that for cells without treatment with the three compounds (green bar; set as 1). All data were normalized to two housekeeping genes and are presented relative to the control (CTR) (set as 1) (means ± SEM from four to eight experiments). *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

As a bifunctional enzyme, the N-terminal domain of ATIC has AICARFT activity, and the C-terminal domain has IMP cyclohydrolase (IMPCH) activity. FA, an IMPCH inhibitor, also promoted HEV replication but exerted cytotoxicity concurrently (Supplementary Figure 2). Thus these results highlight the interaction of nucleotide biosynthesis and the HEV infection process, but also show that rational design of therapy aimed at exploiting the nucleotide biosynthesis pathway for treatment of HEV is not straightforward.

IMPDH inhibition counteracts HEV replication by depleting the purine nucleotide pool

As a branching point in purine synthesis, IMP is converted to either AMP or XMP/GMP (Figure 1A). IMPDH, an enzyme consisting two isoforms (IMPDH1 and IMPDH2) in human, catalyses the reaction of IMP into XMP for further conversion to GMP. We have previously demonstrated that MPA, an clinically used immunosuppressant preferentially inhibiting IMPDH2, has anti-HEV activity [18]. To further explore the potential of targeting this enzyme, a panel of 23 inhibitors were customized designed and synthesized with variable affinities in inhibiting IMPDH1 or IMPDH2 (Supplementary Table 1). As shown in Figure 5A, HEV replication was inhibited by all of the 23 IMPDH inhibitors at concentration of 10 μM measured by luciferase activity. Accordingly, 21 of the 23 inhibitors also supressed HEV infection as assessed by full-length HEV genome quantification by qRT-PCR (Figure 5B). The anti-HEV activity was also observed at 2 μM of 20 IMPDH inhibitors (Supplementary Figure 3) . To further characterize, we selected three representative compounds with anti-HEV activity in both models. Similar to ribavirin and MPA, guanosine supplementation abrogated the anti-HEV activity of these compounds (Figure 5C), suggesting that depletion of the purine nucleotide pool is responsible for their antiviral action. Thus inhibitors with anti-HEV potential exert their action in this respect through targeting nucleotide synthesis.

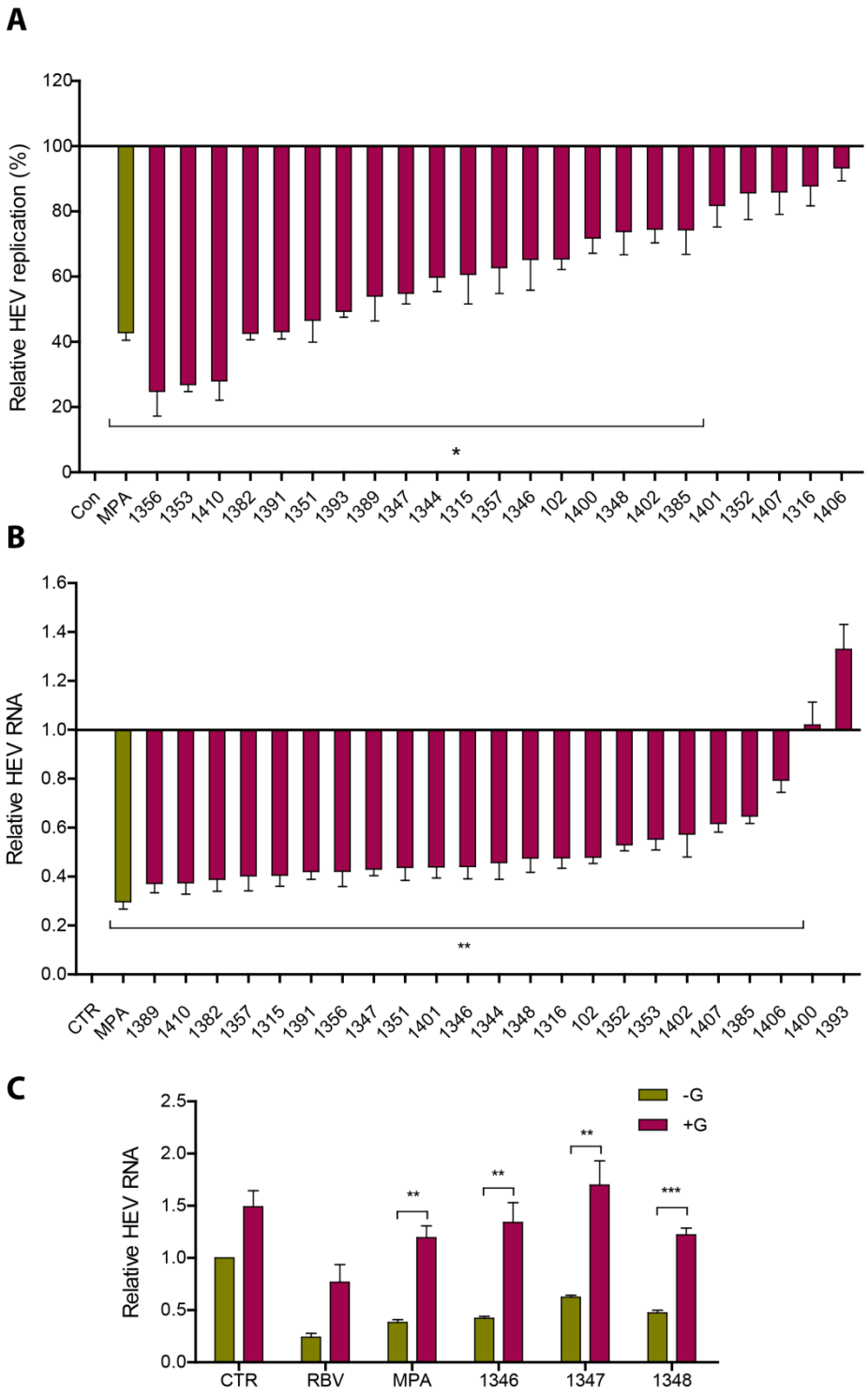

Figure 5. IMPDH inhibitors potently inhibit HEV replication by depletion of the purine nucleotide pool. (A) Huh7 HEV replicon luciferase cells were treated with 23 specific IMPDH inhibitors (10 M) with MPAas a positive control. Luciferase activity was quantified at 24 h after treatment (n = 3) (B) Huh7 cells harbouring full-length HEV were treated with 23 specific IMPDH inhibitors with MPA as a positive control. HEV RNA levels were measured by qRT-PCR at 48 h after treatment (n = 5). (C) Supplementation of guanosine abrogated the anti-HEV effects of 3 representative IMPDH inhibitors (1346, 1347, and 1348) (n = 5). Ribavirin (RBV) and MPA served as positive controls. Data were normalized to two housekeeping genes and are presented relative to the control (CTR) (set as 1) (means ± SEM). *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

Targeting pyrimidine biosynthesis inhibit HEV replication

Even though supplementation of exogenous uridine has no effect on HEV, inhibitors of pyrimidine synthesis have been widely reported to inhibit infection of a broad spectrum of other viruses, prompting further exploration of the role of pyrimidine biosynthesis in HEV replication. We thus selected two catalytic enzymes involved in *de novo* pyrimidine synthesis for further study. Dihydroorotate dehydrogenase (DHODH), which localises to the mitochondria, is a critical enzyme that converts dihydroorotate to orotate. Brequinar (BQR) and leflunomide (LFM) are well-known clinically tested DHODH inhibitors. Treatment with BQR (10 - 500 nM) results in a significant reduction of HEV replication-related luciferase activity in the subgenomic replicon assay system (Figure 6A). Concordantly, BQR also dose-dependently inhibits cellular viral RNA in our infectious HEV model. Treatment with 500 nM BQR for 48 hours resulted in 78 ± 17% (Mean ± SD, n = 7, $P < 0.001$) inhibition of HEV genomic RNA level (determined by qRT-PCR), compared with the control (Figure 6A). Similar results were observed with treatment of LMF (Figure 6B). The specificity of these effects was confirmed in experiments in which we examined by lentiviral RNAi-mediated silencing of the cognate target of these inhibitors, DHODH. Consistently, knockdown of DHODH inhibited HEV replication and abrogated the anti-HEV effect of BQR (Figure 7) and this enzyme does emerges as a relevant target in anti-HEV therapy.

To further identify potential anti-HEV targets, we also examined Orotidine-5'-monophosphate decarboxylase (ODCase), the downstream enzyme of DHODH that catalyses decarboxylation of OMP to UMP. To this end we employed 6-azauracil (6-AU), a potent inhibitor of ODCase. As shown in Figure 6C, HEV replication was dose-dependently inhibited by 6-AU. Conversely, supplementation with uridine fully restored the HEV infectious potential despite the presence of BQR, LMF or 6-AU (Figure.8). In conjunction, these results show that depletion of pyrimidine nucleotide pool is a powerful anti-HEV strategy.

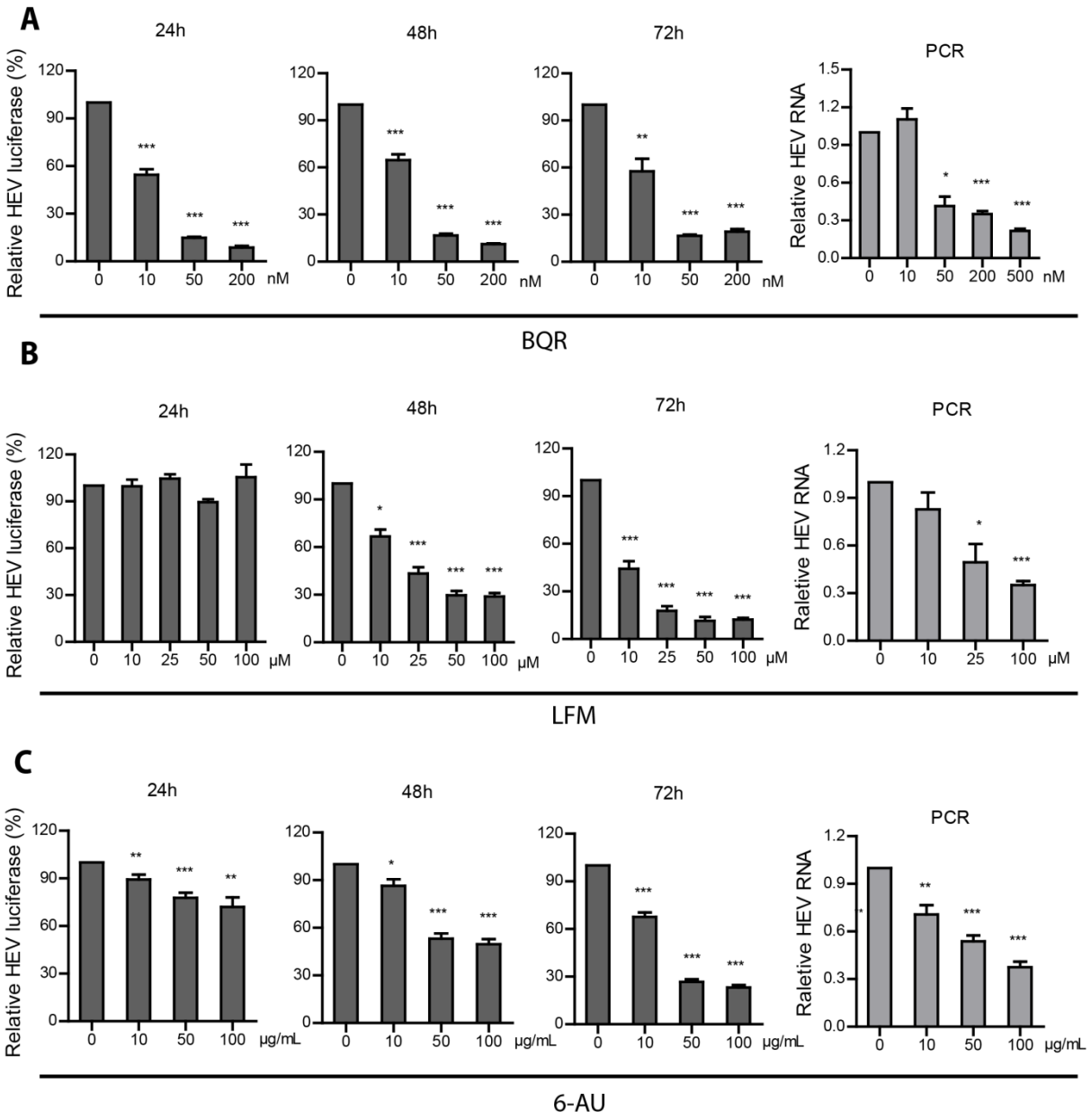

Figure 6. Inhibition of pyrimidine nucleotide synthesis suppresses HEV replication. Huh7 cells containing subgenomic HEV replicons with luciferase report genes were treated with increasing doses of BQR (A), LFM (B), and 6-AU (C). The luciferase activity was determined after 24 h, 48 h, and 72 h. Accordingly, Huh7 cells harboring infectious HEV also were treated with increasing doses of BQR (A), LFM (B), and 6-AU (C). HEV RNA was quantified by qRT-PCR after 48 h of treatment. Data were normalized to two housekeeping genes and are presented relative to the control (CTR) (set as 1). Data represent means ± SEM from four to seven experiments. *, P < 0.05; **, P 0.01; ***, P < 0.001.

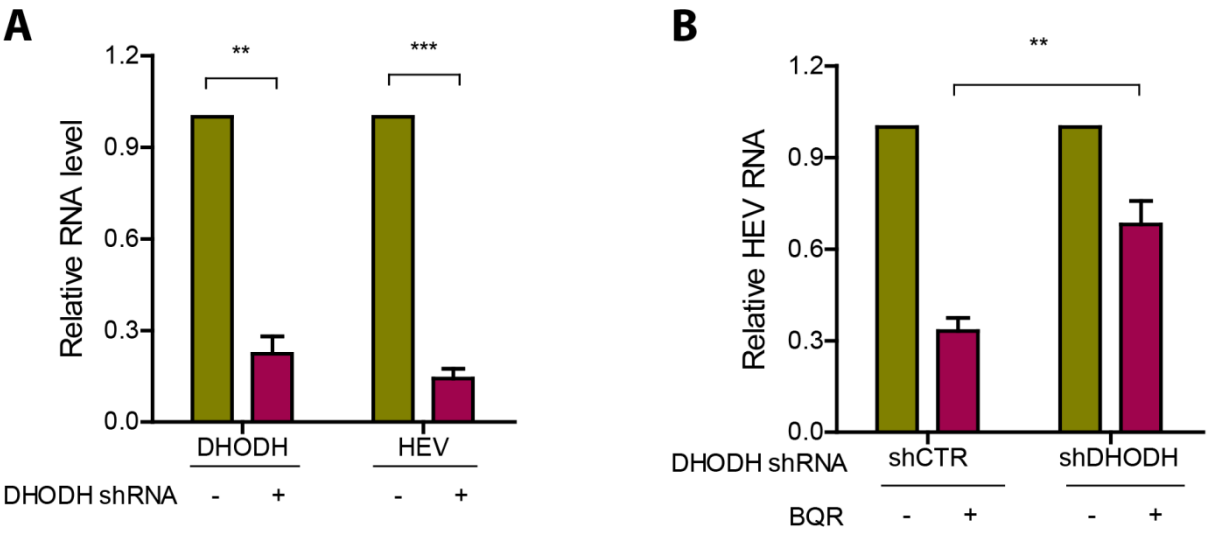

Figure 7. Anti-HEV activity by BQR can be attributed to the inhibition of its target DHODH. (A) Huh7 cells were transduced with lentiviral shRNA to stably silent DHODH (DHODH shRNA+). Huh7 cells transduced with lentiviral shRNA targeting GFP were used as control (DHODH shRNA-). DHODH knockdown was assessed by qRT-PCR (n = 3). DHODH knockdown resulted in significant inhibition of HEV replication. HEV viral RNA were determined 72h after HEV inoculation (n = 6). (B) DHODH knockdown abrogated the anti-HEV effect of BQR (n = 7). Data were normalized to cells without BQR treatment (green bar, set as 1). All data were normalized to two housekeeping genes and presented relative to the control (shCTR) (set as 1) (means ± SEM). **, $P < 0.01$; ***, $P < 0.001$.

Inhibitors of purine and pyrimidine synthesis provoke cellular antiviral immune responses through nucleotide depletion

We previously has demonstrated that the IMPDH inhibitor, MPA, can induce the expression of interferon-stimulated genes (ISGs) to combat hepatitis C virus (HCV) infection, although the underlying mechanism remained unclear [19]. ISGs are the ultimate antiviral effectors and are generally assumed to be induced solely through the action of antiviral cytokines, especially interferons. In HEV infection models, we observed that MPA as well as other (three selected) IMPDH inhibitors were able to induce the expression of a panel of antiviral ISGs (Figure 9A), challenging this dogma. The induction of ISGs by IMPDH inhibitors was associated with purine nucleotide depletion, since supplementation of guanosine at least partly abrogated the induction of ISGs (Figure 9B).

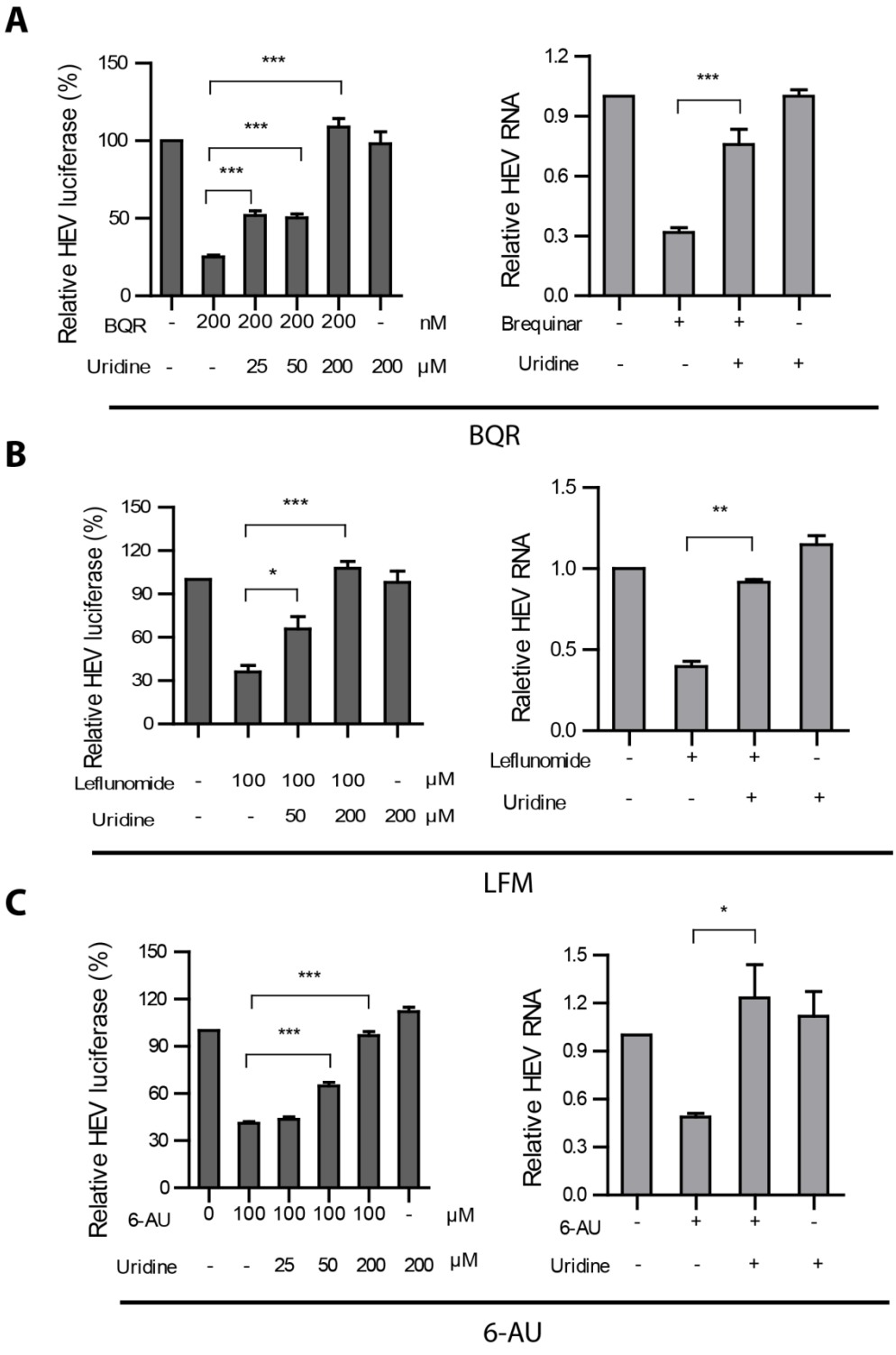

Figure 8. Uridine reverses the anti-HEV activity mediated by pyrimidine inhibition. The Huh7 subgenomic HEV replicon was incubated with BQR (A), LFM (B) and 6-AU (C), supplemented with increasing dose of uridine. After 72h, luciferase activity was determined. Accordingly, Huh7 cells harbouring full-length HEV RNA were treated with BQR (A), LFM (B) and 6-AU (C), and supplemented with 200 µM uridine. HEV viral RNA was assessed by qRT-PCR 48h after treatment. Data were normalized to two housekeeping genes and presented relative to the control (CTR) (set as 1). Data represent mean ± SEM of four to seven experiments. ∗, $P < 0.05$; ∗∗, $P < 0.01$; ∗∗∗, $P < 0.001$.

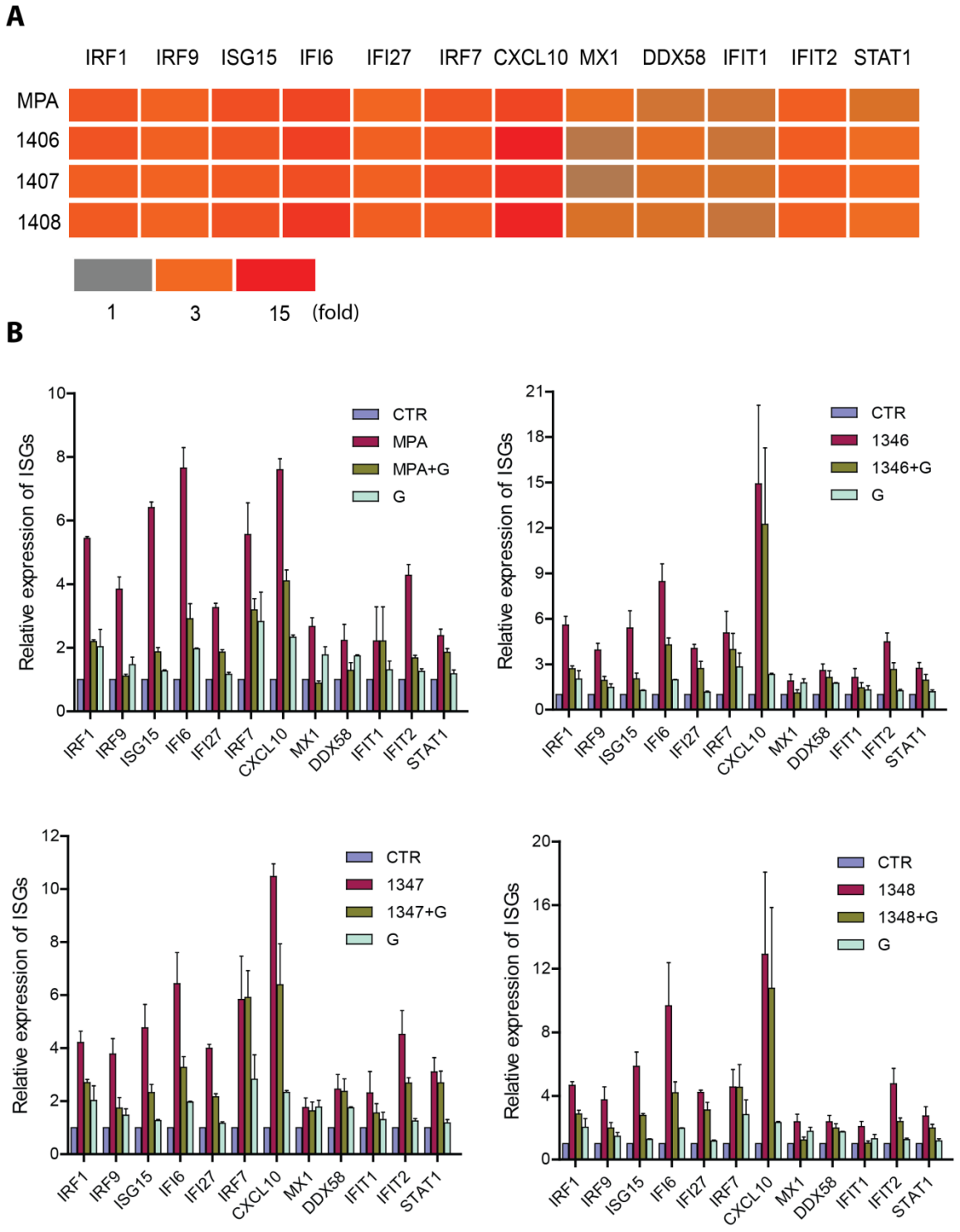

Figure 9. Inhibition of IMPDH stimulates ISG expression through purine nucleotide deprivation. (A) Huh7 cells infected HEV were treated with MPA or 3 other IMPDH inhibitors (1346, 1347 and 1348). The expression of a panel of ISGs were determined by qRT-PCR after 48h treatment. Data were normalized to basal ISG expression without treatment (grey bar, set as 1). (B) Supplementation of guanosine abrogated the induction of ISGs by IMPDH inhibitors. The expression of ISGs were determined by qRT-PCR 48h after treatment. Data were normalized to basal ISG expression without treatment (purple bar, set as 1). All data were normalized to two housekeeping genes and represent means ± SEM of four experiments.

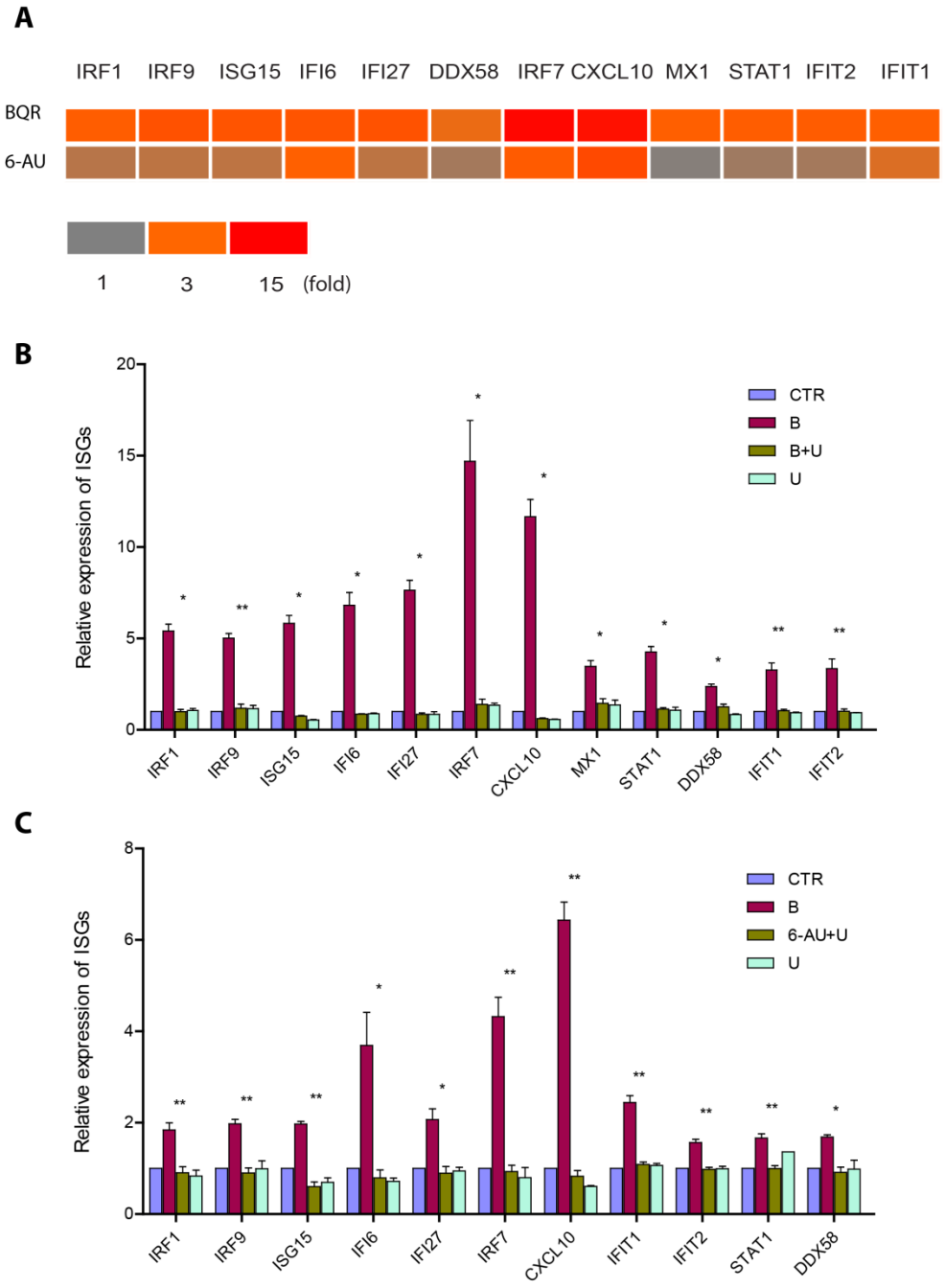

Figure 10. Inhibition of pyrimidine synthesis stimulates ISG expression through pyrimidine nucleotide depletion. (A) Huh7 cells infected with HEV were treated with BQR or 6-AU. After 48h, the expression of a panel of ISGs were determined by qRT-PCR. Data were normalized to basal ISG expression without treatment (grey bar, set as 1). (B) Supplementation of uridine completely abrogated the induction of ISGs by BQR (B) or 6-AU (C).The expression of ISGs were determined by qRT-PCR at 48h after treatment. Data were normalized to basal ISG expression without treatment (purple bar, set as 1). All data were normalized to two housekeeping genes and represent mean ± SEM of five experiments. ∗, $P < 0.05$; ∗∗, $P < 0.01$.

In parallel, we also investigated the effects of pyrimidine synthesis inhibitors. We employed an interferon response reporter that Huh7 cells are stalely integrated with an interferon-stimulated response element (ISRE)-driven luciferase gene that measures ISG transcription upon interferon stimulation. BQR potently induces luciferase activity in this reporter assay, and triggers expression of a panel of ISGs (Supplementary Figure 4 and Figure 10A). Supplementation of uridine completely abrogated these effects on ISG transcription (Figure 10B and Supplementary Figure 4). Similar results were also observed with another pyrimidine synthesis inhibitor, 6-AU, targeting ODCase (Figure 10C). Thus, both purine and pyrimidine synthesis pathways can interact with cellular antiviral immune response, providing a rational explanation as to their antiviral effects.

The induction of ISGs by nucleotide synthesis inhibitors is independent of the JAK-STAT machinery

Classically, ISGs are thought only to be induced by interferons through activation of the JAK-STAT pathway. Briefly, the binding of interferons to their receptors leads to activation of Janus activated kinase 1 (JAK1), resulting in tyrosine phosphorylation of downstream substrates, including signal transducer and activator of transcription 1 (STAT1) and STAT2. The complex of STAT1–STAT2–IRF9 (IFN-regulatory factor 9) enters nucleus and binds to the IFN-stimulated response elements (ISRE) motifs in the target gene, subsequently regulating ISG transcription and thus mediating the innate anti-viral immune response.

To assess whether the induction of ISGs by nucleotide synthesis inhibitors also occurs via this classical pathway, we blocked JAK-STAT cascade by employing the pharmacological JAK inhibitors, JAK inhibitor 1 or CP-690550, which were conceivably identified to impair the expression ISGs triggered by IFN-α (Supplementary Figure 5). Surprisingly, the induction of ISGs as well as the anti-HEV effects of these inhibitors were not affected (Figure 11). These results revealed that targeting nucleotide synthesis provokes ISG induction via a non-canonical mechanism that is independent of the classical interferon signalling.

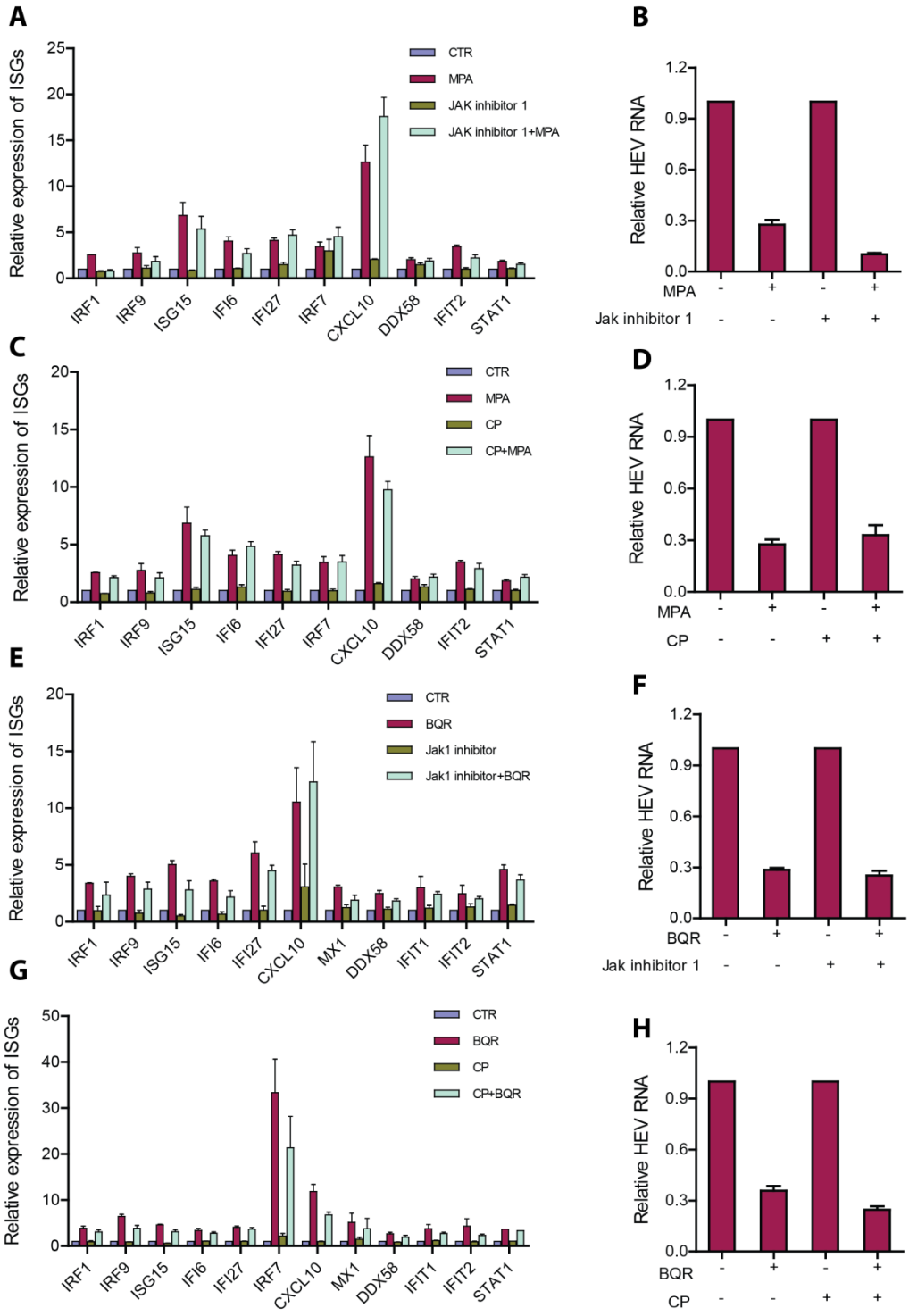

Figure 11. ISGs induction and the anti-HEV activity triggered by nucleotide synthesis inhibitors are independent of the JAK-STAT signalling pathway. The induction of ISGs (A, C) and the anti-HEV effects (B,D) by MPA were quantified in presence or absence of JAK inhibitor 1 (A, B)/CP-690550 (CP) (C, D); The induction of ISGs were normalized to basal ISG expression without MPA treatment (purple bar, set as 1). The relative HEV RNA levels were normalized to cells without treatment of MPA (set as 1). Similarly, the induction of ISGs (E, G) and the anti-HEV effects (F, H) mediated by BQR were quantified in presence or absence of JAK inhibitor 1 (E, F)/CP (G, H). The induction of ISGs were normalized to basal ISG expression without BQR treatment (purple bar, set as 1). The relative HEV RNA levels were normalized to cells without BQR treatment (set as 1). Data were normalized to two housekeeping genes and represent means ± SEM of three to four experiments.

Discussion

Nucleotides are key components involved in host cell metabolism and virus infection. Most of the inhibitors targeting *de novo* nucleotide biosynthesis have been well-characterized by many studies and their efficacy in inhibiting nucleotide synthesis have been thoroughly demonstrated [16,17,24-31]. Based on that, we profiled and established the effects and mechanism-of-action of inhibiting *de novo* nucleotides biosynthesis on HEV replication. Unexpectedly, targeting the early steps of the purine nucleotide synthesis pathway (before the primary purine IMP formed) leads to enhancement of HEV replication, whereas targeting later steps (IMPDH enzyme) results in potent antiviral activity against HEV, an effect apparently relating to purine nucleotide depletion. Inhibition of pyrimidine nucleotide synthesis pathway also inhibits HEV replication. Mechanistically, these effects are related to an unconventional interaction with cell-autonomous antiviral immunity dependent on very strong induction of antiviral ISGs.

It is counterintuitive that targeting the upstream enzymes of the purine pathway (before IMP formed) by pharmacological inhibitors facilitates HEV replication, but the specificity became evident from silencing genes encoding the enzymes involved. Supplementation with exogenous purine nucleotides (adenosine or guanosine) in culture medium in presence of these purine synthesis inhibitors were not capable of abrogating the stimulation of HEV replication, suggesting these pro-viral effects may only partly relate to the nucleotide synthesis pathway (Supplementary Figure 6A-C). It is worth noting that targeting the early stage of purine synthesis result in depletion of ATP and/or GTP pool. Cellular energy metabolism mediated by ATP might be important for the host cells to defend virus infection [32,33]. Therefore, insufficient ATP level might facilitate HEV infection by escaping from host cellular immunity. However, how the ATP levels regulate virus infection deserves further investigation. Similarly, a previous study reported pro-viral activity by nucleotides biosynthesis inhibitors, LFM and FK778, in hepatitis B virus model, although these two compounds are generally antiviral against other viruses [17]. Thus, the question whether the pro-HEV effects of targeting the early steps of the purine pathway are specific to this virus or a general phenomenon in virus biology remains unanswered.

IMPDH, as a target for antiviral drug development for a broad spectrum of viruses, has been widely investigated. We previously have demonstrated that the IMPDH inhibitors ribavirin and MPA inhibit HEV replication *in vitro* [18,20]. This study further validated this notion by showing the anti-HEV potential of 23 specifically designed IMPDH inhibitors. The efficacy of 23 IMPDH inhibitors on HEV infection were consistent but with variable degree, which might be due to the different ability and variable affinities in inhibiting IMPDH1 and IMPDH2. As a competitive IMPDH inhibitor, ribavirin has been used in the clinic to treat chronic hepatitis C for decades. However, ribavirin monotherapy hardly has detectable effect on HCV viral load reduction [34], but only when combined with IFN-α, it doubles the response rate, compared with IFN-α alone [35]. In contrast, ribavirin monotherapy as off-label treatment appears very effective for treating chronic HEV infection in that viral clearance was observed in the majority of the patients as reported by a recent large retrospective multicentre study [36], although prospective randomized trials are still required to confirm the findings. Of note that in addition to IMPDH inhibition, ribavirin also possesses pleiotropic biological properties, including immunomodulation, inhibition of gene translation, and interaction with viral RNA-dependent RNA polymerase (RdRp) and mutation of virus [37-39]. Thus, the exact anti-HEV mechanism by ribavirin remains to be further elucidated, but the present study provides evidence that answer may lie in its relation to nucleotide biosynthesis.

As a non-competitive IMPDH inhibitor, MPA has been used as an immunosuppressant to prevent allograft rejection following organ transplantation [40]. Despite of the opposing effects of inhibitors targeting early or later steps of purine synthesis cascade on HEV, we demonstrated that the anti-HEV action of MPA was independent of those early step enzymes (Supplementary Figure 6D) Interestingly, clinical evidence appears to support our experimental observation that the use of immunosuppressive treatments containing mycophenolate mofetil (the pro-drug of MPA) may lead to more frequent HEV clearance in heart transplant recipients [41]. Nevertheless, because of limited patient number, it is still not sufficient to draw solid conclusion regarding the *in vivo* effect of MPA. A recent cohort study reported the anti-HEV activity by ribavirin was not affected by MPA in patients, but they didn't analyse the direct effect of MPA on HEV infection [42].

The three inhibitors used in our study interfering pyrimidine synthesis have been described in many previous studies [16,29-31]. Adding to the previous knowledge that pyrimidine synthesis inhibitors, such as BQR and LFM, have broad antiviral activity against a spectrum of viruses [16,23,43], we now report their potent anti-HEV activity. Both BQR and LFM are immunosuppressive agents, although whether the mechanism of action is solely via pyrimidine inhibition remains controversially unclear [44-46]. The efficacy of BQR against graft rejection has been extensively investigated in preclinical models [47-49]; whereas LFM has been proposed as off-label immunosuppressive therapy in bone marrow [11] and renal [50] transplantation. In addition, DHODH inhibitors have been explored to treat various other diseases, including malaria, autoimmune and inflammatory diseases, cancer, rheumatoid arthritis and psoriasis [51-55]. Given the bifunctional effects of antiviral and immunosuppressive of BQR and LFM, these regimens may hold the potential to treat HEV-infected organ recipients.

Interestingly, nucleotide synthesis interacts with cellular antiviral immune responses. Here we demonstrated a direct effect of depletion of nucleotide pools on the transcription of antiviral ISGs. ISGs are ultimate antiviral effectors that are thought to be induced by interferons only. Although hundreds of ISGS have been identified, recent functional studies of individual ISG have surprisingly found out that only a small subset of ISGs actually have potent or broad antiviral activities, which include IRF1, DDX58 and IRF7 [56,57]. It is these antiviral ISGs that are induced in our HEV models upon treatment with nucleotide synthesis inhibitors. Consistently, previous studies in HCV models reported that induction of IRF1 or IRF7 was associated with the antiviral activity of MPA [19] or ribavirin [58], respectively. Furthermore, the antiviral activity of inhibitors of pyrimidine biosynthesis against measles virus, chikungunya virus and West Nile virus was also associated with the induction of ISGs [23].

For now the mechanistic details as to inhibitors of nucleotide biosynthesis can induce ISGs remain obscure. Classically, transcription of ISGs is initiated from the binding of interferons to their receptors, which subsequently drives the activation of JAK-STAT cascade [56]. Inhibition of JAK1 to phosphorylate STAT1, the key event of interferon signalling transduction, often results in complete blockage of antiviral interferon responses [59]. However, exceptions also exist in that ISGs can be induced in the absence of JAK1 or STAT1 activation [60,61]. Here, we found that induction of ISGs and the anti-HEV effects by nucleotide

synthesis inhibitors are independent of the classical JAK-STAT cascade, suggesting the involvement of a non-canonical mechanism that is independent of interferons and identification of these mechanisms should have substantial value for our understanding of antiviral immunity.

In conclusion, selectively targeting host enzymes involved in *de novo* nucleotide biosynthesis potently inhibits HEV replication. Furthermore, nucleotide biosynthesis pathways interact with cellular immune response that all the pharmacological inhibitors exerting anti-HEV activity are capable of triggering antiviral ISG transcription. Thus, targeting nucleotide biosynthesis represents a viable option for antiviral drug development against HEV.

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Supplementary Materials

Supplementary Table 1. Information of 23 specific IMPDH inhibitors

CDD-KP-#	IMPDH I Ki in nM	IMPDH 2 Ki in nM
102	330	250
1316	82.10	55,70
1315	0.60	13.90
1401	2553	231.1
1344	162.80	102.40
1346	859.1	243.9
1347	703.4	230.1
1348	1725	365,7

CDD-KP-#	IMPDH I Ki in nM	IMPDH 2 Ki in nM
1351	618.40	185.90
1352	136.20	93.01
1353	1255	364.70
1356	70.12	65.69
1410-L-ABC	N/D	N/D
1357	1813	552.50
1389	11274	297.90
1391	4221	2160

CDD-KP-#	IMPDH I Ki in nM	IMPDH 2 Ki in nM
1393	5556	2398
1400	9138	367.7
1402	2203	146.7
1406	N/D	N/D
1407	N/D	N/D
1382	1815	2154
1385	521.80	487.40

Inhibitory constant (Ki) on IMPDH1 and IMPDH2 of 23 specific IMPDH inhibitors. First 3 compounds in the table are insoluble in DMSO. Other compounds are soluble in DMSO. Compounds 1406 and 1407 do not inhibit IMPDH but if converted in the cell into their corresponding NAD analogues should show some inhibition of the enzymes. Ki of 1410-L-ABC, 1406 and 1407 was not determined (ND). Chemical structures of the IMPDH inhibitors will be published somewhere else.

Supplementary Table 2. Primer sequences

Gene	Sequences 5' to 3'
HEV-F	ATTGGCCAGAAGTTGGTTTTCAC
HEV-R	CCGTGGCTATAATTGTGGTCT
DDX58-F	CACCTCAGTTGCTGATGAAGGC
DDX58-R	GTCAGAAGGAAGCACTTGCTACC
ISG15-F	CTCTGAGCATCCTGGTGAGGAA
ISG15-R	AAGGTCAGCCAGAACAGGTCGT
STAT1-F	ATGGCAGTCTGGCGGCTGAATT
STAT1-R	CCAAACCAGGCTGGCACAATTG
IFI27-F	CGTCCTCCATAGCAGCCAAGAT
IFI27-R	ACCCAATGGAGCCCAGGATGAA
IRF1-F	GAGGAGGTGAAAGACCAGAGCA
IRF1-R	TAGCATCTCGGCTGGACTTCGA
IRF9-F	CCACCGAAGTTCCAGGTAACAC
IRF9-R	AGTCTGCTCCAGCAAGTATCGG
IFIT1-F	GCCTTGCTGAAGTGTGGAGGAA
IFIT1-R	ATCCAGGCGATAGGCAGAGATC
IFIT2-F	GGAGCAGATTCTGAGGCTTTGC
IFIT2-R	GGATGAGGCTTCCAGACTCCAA
IFI6-F	TGATGAGCTGGTCTGCGATCCT
IFI6-R	GTAGCCCATCAGGGCACCAATA
IRF7-F	CCACGCTATACCATCTACCTGG
IRF7-R	GCTGCTATCCAGGGAAGACACA
CXCL10-F	GGTGAGAAGAGATGTCTGAATCC
CXCL10-R	GTCCATCCTTGGAAGCACTGCA
MX1-F	GGCTGTTTACCAGACTCCGACA
MX1-R	CACAAAGCCTGGCAGCTCTCTA
APRT-F	GCGATTGAAGCACCTGTGGATG
APRT-R	CGGTTTTTACACAGCACCTCCAC
GART-F	GCACATCTCTGCCTGTTTGGCT
GART-R	CATGGAACACCTCCAGTCCTAG
ATIC-F	CCGAGAGTAAGGACACCTCCTT
ATIC-R	GGCATCTGAGATACGCCTTTGC
DHODH-F	GAGGACATTGCCAGTGTGGTCA
DHOSH-R	TTCCCACTCAGCCCTCCTGTTT

Supplementary Table 3. shRNA sequences

No.	Gene	ACCESSION	Sequences	Target Sequence
APRT	PPAT (3)	NM_002703.3	CCGGCCCTTCGTTGTTGAAACACTTCTCGAGAAGTGTTTCAACAACGAAGGGTTTTTG	CCCTTCGTTGTTGAAACACTT
GART sh1	GART (1)	NM_000819.3	CCGGGCCCAGGAGTTTGACTTACAACTCGAGTTGTAAGTCAAACTCCTGGGCTTTTTG	GCCCAGGAGTTTGACTTACAA
GART sh2	GART (2)	NM_000819.3	CCGGGCACAGTCTCATCATGTCAAACTCGAGTTTGACATGATGAGACTGTGCTTTTTG	GCACAGTCTCATCATGTCAAA
GART sh3	GART (3)	NM_000819.3	CCGGCCCTAACTGTTGTCATGGCAACTCGAGTTGCCATGACAACAGTTAGGGTTTTTG	CCCTAACTGTTGTCATGGCAA
ATICsh1	ATIC (1)	NM_004044.4	CCGGGCCTTGACAATACTTTCCAAACTCGAGTTTGGAAAGTATTGTCAAGGCTTTTTG	GCCTTGACAATACTTTCCAAA
ATIC sh2	ATIC (2)	NM_004044.4	CCGGGCAATCTCTATCCCTTTGTAACTCGAGTTACAAAGGGATAGAGATTGCTTTTTG	GCAATCTCTATCCCTTTGTAA
ATIC sh3	ATIC (3)	NM_004044.4	CCGGGCTGGAATCCTAGCTCGTAATCTCGAGATTACGAGCTAGGATTCCAGCTTTTTG	GCTGGAATCCTAGCTCGTAAT
DHODH	DHODH (5)	NM_001361.3,NM_001025193.1	CCGGGTGAGAGTTCTGGGCCATAAACTCGAGTTTATGGCCCAGAACTCTCACTTTTT	GTGAGAGTTCTGGGCCATAAA

Supplementary Figure 1

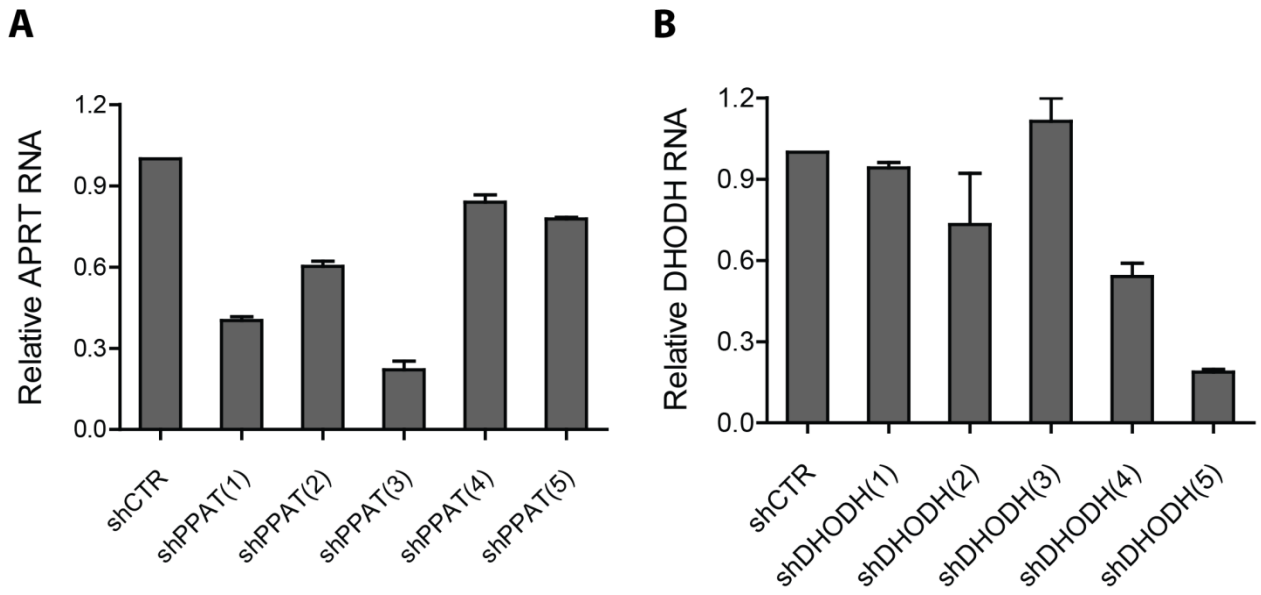

Figure S1. Gene knockdown of PPAT and DHODH with different shRNA lentiviral vectors. Huh7 cells were transduced with lentiviral shRNAs targeting at PPAT (A) and DHODH (B) using a set of 5 different independent shRNA preparations for each gene knockdown. Huh7 cells transduced with lentiviral shRNA targeting GFP (shCTR) were used as control. The efficiency of knockdown of the 2 genes were analysed by qRT-PCR using specific primers. Data were normalized to GAPDH and presented relative to control (set as 1).

Supplementary Figure 2

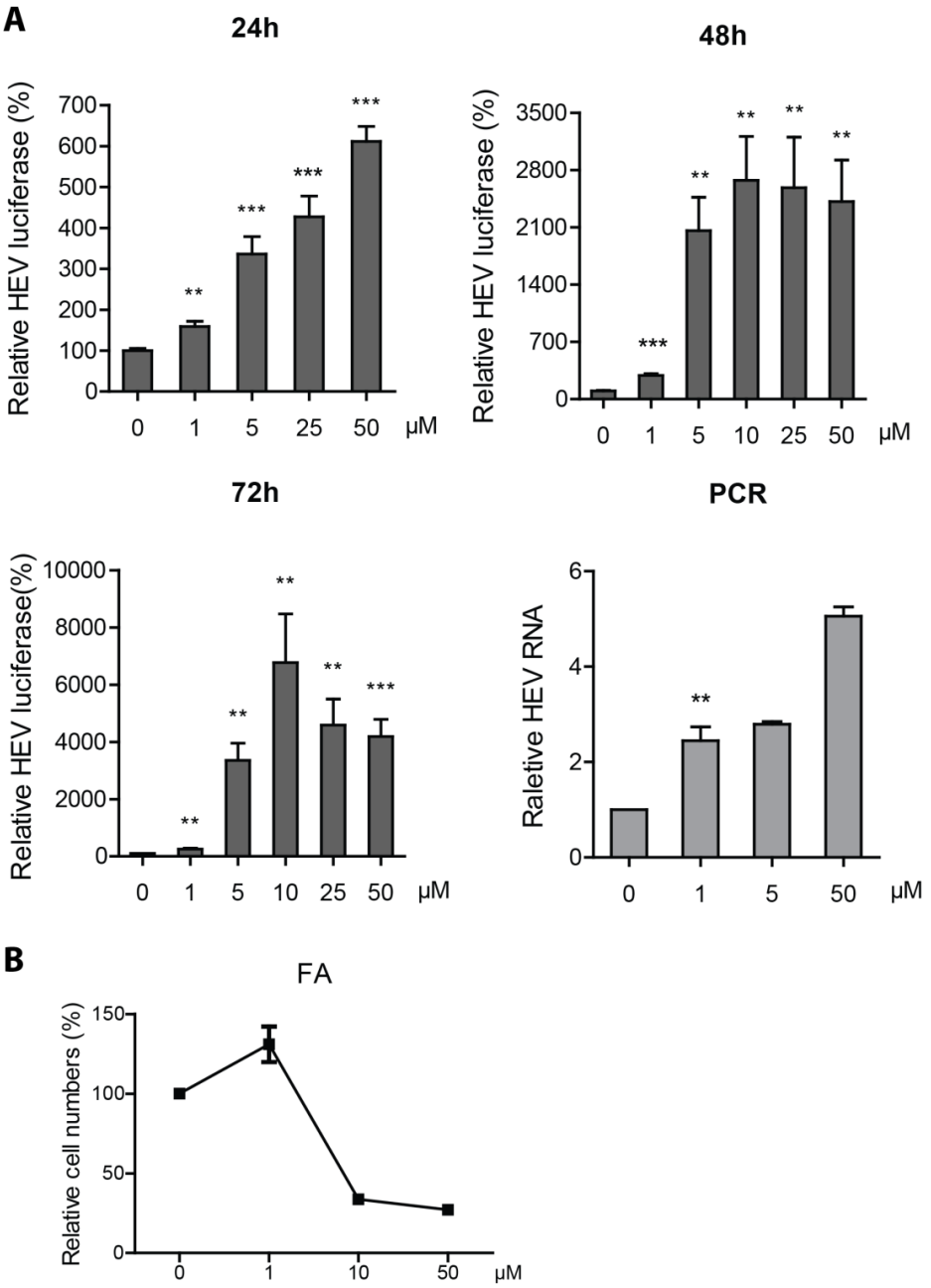

Figure S2. Fludarabine enhances HEV replication. (A) Huh7 cell-based subgenomic HEV replicon containing the luciferase reporter gene were treated for 24h, 48h and 72h with a dose-range of FA. Data presented as means ± SEM. Meanwhile, Huh7 cells with the infectious HEV containing the full-length p6 genome were treated for 48h with a dose-range of FA. Data were normalized to GAPDH and presented relative to results from untreated cells (set as 1). (B) Huh7 cells were incubated with dose-range of FA. After 72h, MTT assay was performed to determine cytotoxicity of FA.

Supplementary Figure 3

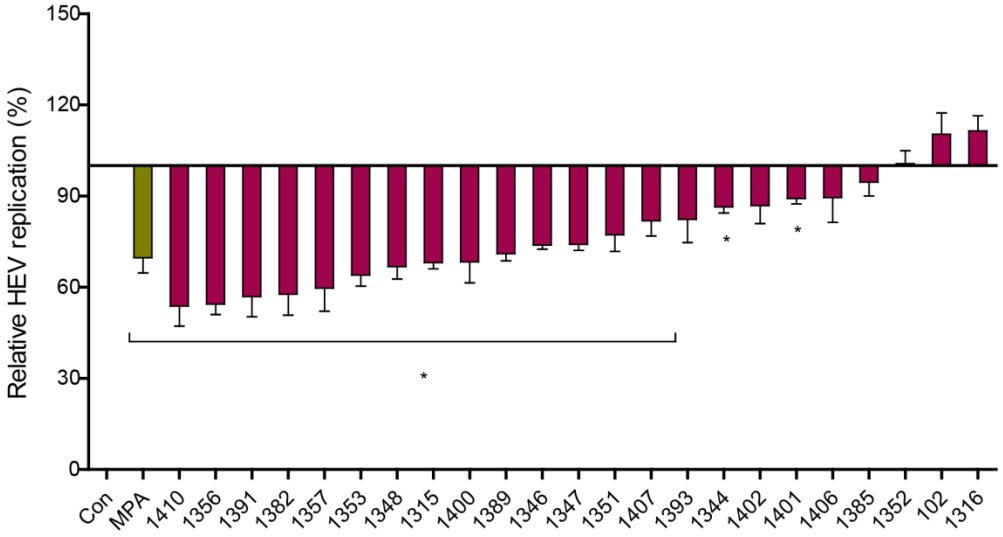

Figure S3. 2 µM IMPDH inhibitors moderately inhibit HEV replication. Huh7 HEV replicon luciferase cells were treated with 23 specific IMPDH inhibitors (2µM) with MPA as a positive control. Luciferase activity was quantified at 24h after treatment (n = 3).

Supplementary Figure 4

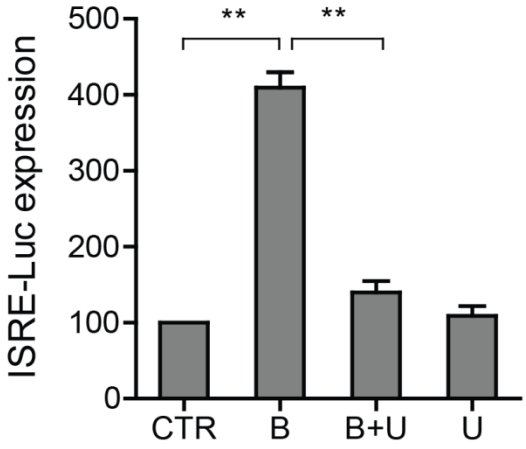

Figure S4. BQR stimulates ISRE transcription through pyrimidine depletion. Huh7-ISRE-Luc cells were incubated with BQR (B) in presence or absence of Uridine (U). ISRE promoter-related firefly luciferase activity was quantified 72h after culture.

Supplementary Figure 5

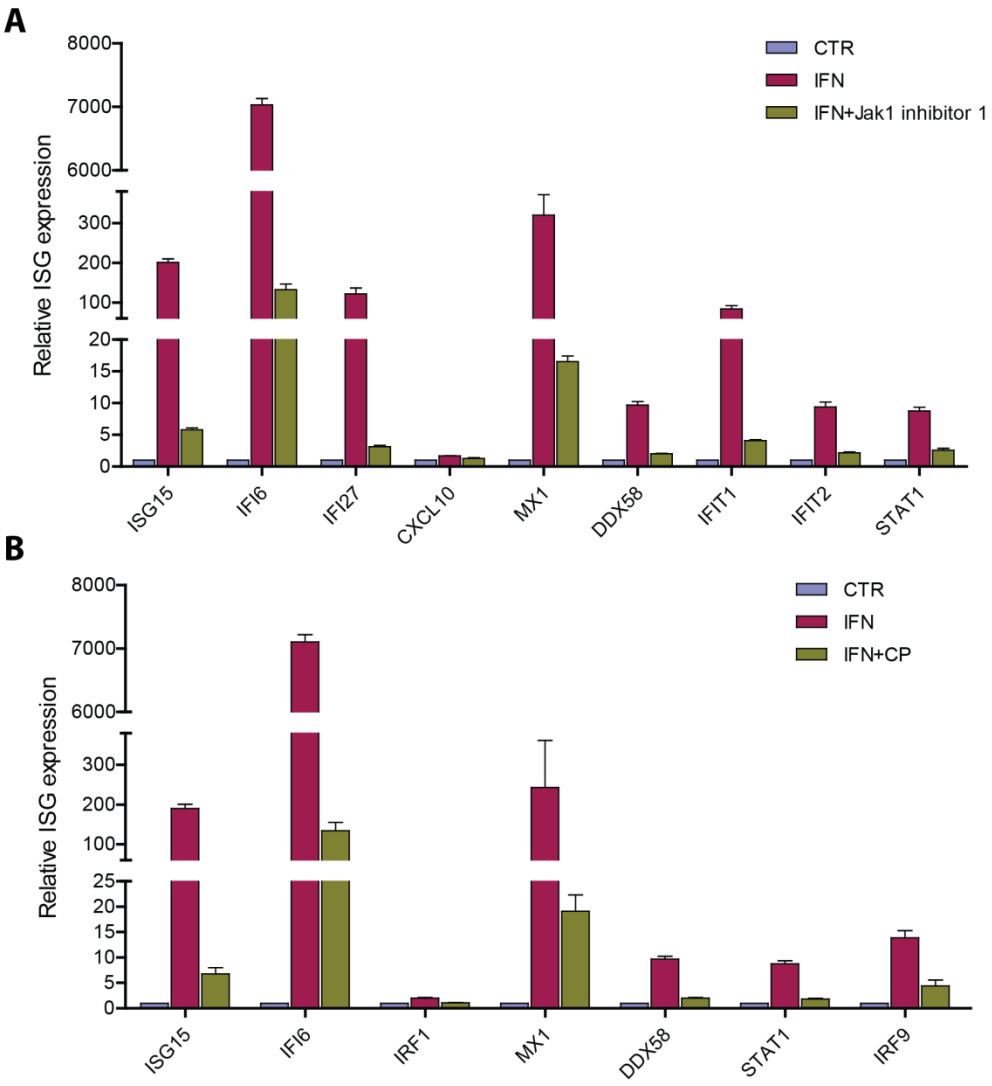

Figure S5. JAK inhibitors diminish IFNα stimulated ISG expression. (A) Huh7 cells infected with HEV were incubated with IFNα in presence or absence of Jak inhibitor 1. After 48h, the expression of ISGs were assessed by qRT-PCR. (B) Same experiment was performed with another JAK inhibitor, CP-690550 (CP). Data were normalized to basal ISG expression without IFNα treatment (purple bar, set as 1). All data were normalized to two housekeeping genes and experiments were performed two to five times.

Supplementary Figure 6

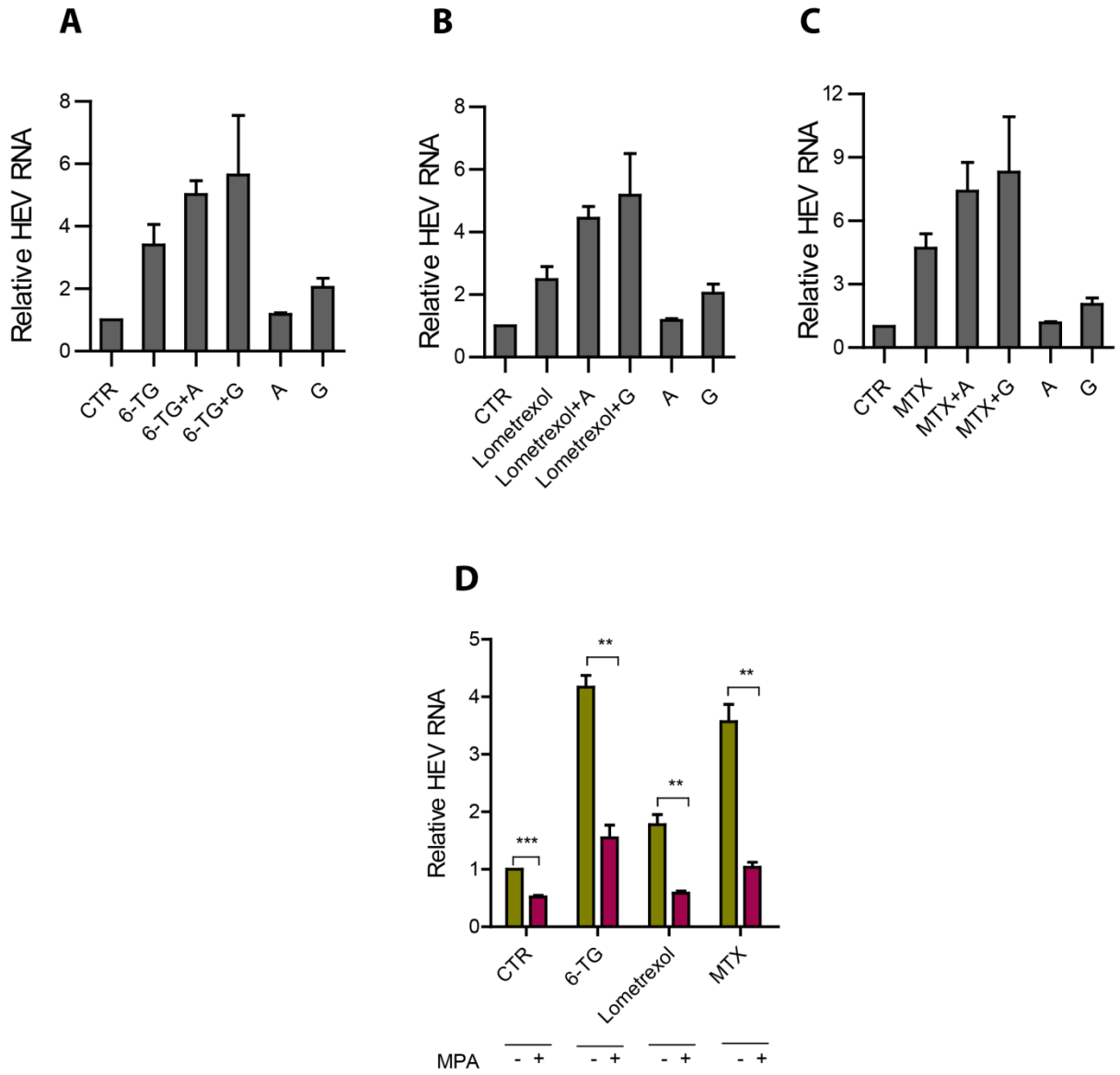

Figure S6. Inhibition of IMP synthesis enhanced HEV replication independent of purine depletion and does not affect the anti-HEV activity of MPA. Huh7 cells infected HEV were treated with 6-TG (A), lometrexol (B) and MTX (C), respectively, in the presence or absence of guanosine (G)/adenosine (A). The HEV RNA was assessed by qRT-PCR 48h after treatment. (D) Huh7 cells infected HEV were treated with 6-TG, lometrexol and MTX, respectively, in the presence or absence of MPA. The HEV RNA was assessed by qRT-PCR 48h after treatment. Data were normalized to two housekeeping genes and presented relative to the control (CTR) (set as 1). Experiments were performed two to five times.

Supplementary Figure 7

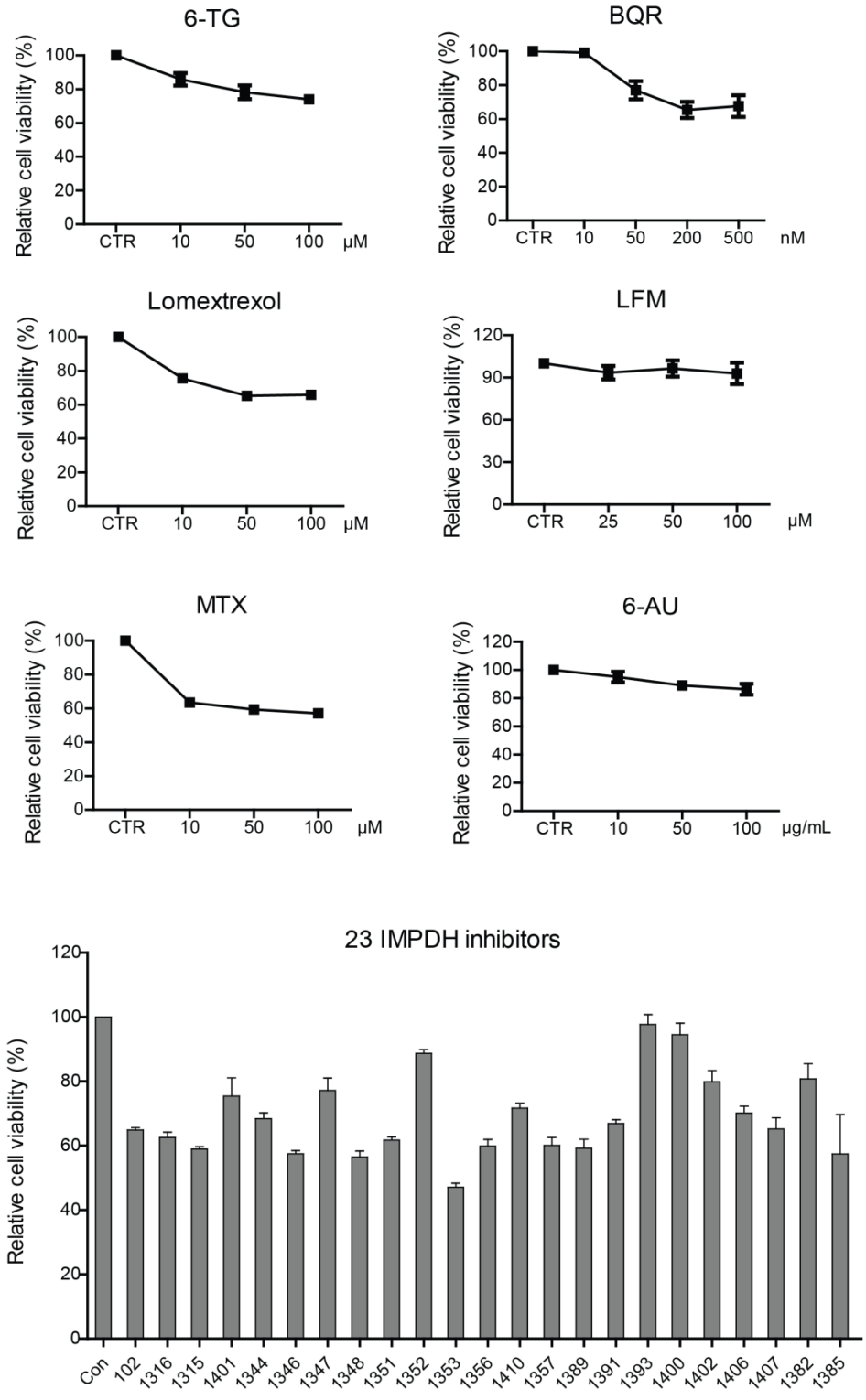

Figure S7. The effects of nucleotide synthesis inhibitors on Huh7 cells viability. Huh7 cells were incubated with a dose-range of 6-TG, lometrexol, MTX, BQR, LFM or 6-AU for 72h treatment. Huh-7 cells were incubated with 23 IMPDH inhibitors at 10 µM for 48h. MTT assay was performed to determine cytotoxicity of these compounds.

CHAPTER 7

Protein kinase C alpha functions as the key host factor to constrain hepatitis E virus

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Submitted.

Abstract

Although hepatitis E has emerged as a global health issue, there is no approved medication and limited knowledge of its infection biology. Aiming to investigate the role of protein kinases in hepatitis E virus (HEV) infection and to identify potential antiviral targets, we screened a library of pharmacological kinase inhibitors on a state-of-art cell culture model: a subgenomic HEV replicon containing luciferase reporter. After screening, we identified protein kinase C alpha (PKCα) as an essential cell host element for defense against HEV replication. Both specific conventional PKC inhibitor and functional knockdown enhanced HEV replication. Whereas the functional over-expression or pharmacologic activator strongly inhibited HEV replication. Importantly, upon the stimulation of its activator, PKCα can efficiently activate its downstream NF-κB or AP-1 pathways. However, PKCα induced HEV inhibition is totally independent of these two downstream pathways. The discovery that activated PKCα restricts HEV replication provides a novel target for managing HEV infection.

Keywords: PKCα, hepatitis E virus, PMA, NF-κB, AP-1

Introduction

Hepatitis E virus (HEV) is one of the most common causes of acute viral hepatitis in the world. Although the mortality rate is < 1% among the general population, pregnant women can have a fatality rate of up to 30%. Additionally, chronic hepatitis E has increasingly become a significant clinical problem in immunocompromised patients. Up to date, there is still no proven medication available and its infection biology is poorly understood.

Protein kinases are principal components of the machineries that orchestrate immune response against diverse pathogenic entities, including viruses, by subsequent stimulation of specific signal transduction cascades [1]. However, kinase controlled pathways employed by the host cell to stimulate antiviral immunity remain largely obscure. Knowledge of such pathways could prove exceedingly useful for the rational design of therapeutic avenues against HEV infection.

Encouragingly, numerous pharmacological kinase inhibitors or activators have been developed to target particular kinases. Among those, several are approved drugs in particular for treating cancer [2], and many are currently at various stages of preclinical and clinical development. These compounds have broad implications for treating various diseases, including cancer, inflammation, diabetes and viral infections [3,4].

Thus, this study aims to comprehensively profile kinase-mediated cascades in cell–autonomous antiviral immunity starting from screening a library of pharmacological kinase inhibitors on Huh7 based HEV replication cell model. We identified protein kinase C alpha (PKCα) as an important anti-HEV mediator. Hence, our results defined PKCα as a novel antiviral element in the machinery combatting HEV infection.

Materials and Methods

Pharmacological kinase inhibitors

The kinase inhibitor library used for the screening was made available by the KU Leuven Centre for Drug Design & Development (www.cd3.eu).

Reagents and Antibodies

Stocks of PMA (Sigma-Aldrich, St Louis, MO) and JAK inhibitor I (Santa Cruz Biotech, Santa Cruz, CA) were dissolved in dimethyl sulfoxide (DMSO) (Sigma-Aldrich, St Louis, MO) to concentrations of 100 µg/ml and 20 mM, respectively. Antibodies including phospho-PKCα/β (#9375), c-Fos (9F6, #2250), RelA (C22B4, #4764), Anti-rabbit IgG(H+L),F(ab') 2 Fragment (Alexa Fluor 488 conjugate) and Anti-mouse IgG (H+L), F(ab')2 Fragment (Alexa Fluor® 488 Conjugate) were purchased from Cell Signaling Technology, the Netherlands. Anti-rabbit or anti-mouse IRDye-conjugated antibodies were used as secondary antibodies for western blotting (Stressgen, Victoria, BC, Canada).

Viruses and cell culture models

Hepatocellular carcinoma cells Huh7 were kindly provided by Professor Bart Haagmans from Department of Viroscience, Erasmus Medical Center. Human Embryonic Kidney 293 cells were originally obtained from ATCC (www.atcc.org). The HEV infectious model was based on Huh7 cells containing the full-length HEV genome (Kernow-C1 p6 clone, GenBank Accession Number JQ679013). Infectious HEV particles are generated and secreted into cell culture medium, which can be collected and used for secondary infection [5-8]. The HEV subgenomic model was based on Huh7 cells containing the subgenomic HEV sequence (Kernow-C1 p6/luc) coupled to a Gaussia luciferase reporter gene. Luciferase normalization cells (Huh7-norm) were generated by transducing Huh7 cells with a lentiviral vector expressing the firefly luciferase gene under control of the human phosphoglycerate kinase (PGK) promoter. NF-κB, AP-1 luciferase reporter cells were generated by transducing Huh7 cells with lentiviral vectors expressing the firefly luciferase gene under the control of NF-κB, AP-1 promoters, respectively (System Biosciences).

Screen of pharmacological kinase inhibitors

After optimizing the screening protocol, a concentration of 0.8 µM was used to treat Huh7 cells transfected with HEV p6/luc replicon RNA for 72 hrs, followed by measurement of luciferase activity, as described previously [9]

Gene knockdown or over-expression by lentiviral vectors

Lentiviral pLKO knockdown vectors (Sigma–Aldrich) targeting PKCα, PKCβ, RelA, c-Fos or control were obtained from the Erasmus Biomics Center and produced in HEK293T cells as previously described [10]. After a pilot study, the shRNA vectors exerting optimal gene knockdown were selected. These shRNA sequences are listed in Supplementary Table 2. Stable gene knockdown cells were generated after lentiviral vector transduction and puromycin (2.5μg/ml; Sigma) selection.

wtPKCα and caPKCα overexpression lentivral vectors were a kind gift from Dr. Lin from the University of Minnesota. To create a stable overexpression cell lines, following lentiviral vectors transduction, cell cytometry and FACS were used to detect and selectively pick up the positive cells by the GFP tag gene expression on the vector.

Measurement of luciferase activity

For Gaussia luciferase, the secreted luciferase activity in the cell culture medium was measured by BioLux® Gaussia Luciferase Flex Assay Kit (New England Biolabs). For firefly luciferase, luciferin potassium salt (100 mM; Sigma) was added to cells and incubated for 30 min at 37 °C. Both Gaussia and firefly luciferase activity was quantified with a LumiStar Optima luminescence counter (BMG LabTech, Offenburg, Germany).

Quantitative real-time polymerase chain reaction

RNA was isolated with a Machery-NucleoSpin RNA II kit (Bioke, Leiden, The Netherlands) and quantified using a Nanodrop ND-1000 (Wilmington, DE, USA). cDNA was synthesized from total RNA using a cDNA Synthesis Kit (TAKARA BIO INC). The cDNA of all detected genes was amplified for 50 cycles and quantified with a SYBRGreen-based real-time PCR (Applied Biosystems) according to the manufacturer's instructions. GAPDH was considered as reference genes to normalize gene expression. Relative gene expressions were normalized to GAPDH using the formula $2^{-\Delta\Delta CT}$ ($\Delta\Delta CT = \Delta CT_{sample} - \Delta CT_{control}$). All the primer sequences are included in Supplementary Table 3.

Western Blot Assay

Cultured cells were lysed in Laemmli sample buffer containing 0.1 M DTT and heated 5 mins at 95 °C, followed by loading onto a 10% sodium dodecyl sulfate polyacrylamide gel

and separation by electrophoresis. After 90 mins running at 120 V, proteins were electrophoretically transferred onto a polyvinylidene difluoride membrane (Invitrogen) for 1.5 hrs with an electric current of 250 mA. Subsequently, the membrane was blocked with a mixture of 2.5 ml blocking buffer (Odyssey) and 2.5 ml phosphate-buffered saline containing 0.05% Tween 20. It was followed by overnight incubation with primary antibodies (1:1000) at 4 °C. The membrane was washed 3 times followed by incubation for 1h with IRDye-conjugated secondary antibody (1:5000). After washing 3 times, protein bands were detected with the Odyssey 3.0 Infrared Imaging System.

Confocal laser electroscope assay

Huh7 cells were seeded on glass coverslips. After 12 hrs, cells were washed with PBS, fixed in 4% formalin for 10 mins and blocked with tween-milk-glycine medium (PBS, 0.05% tween, 5g/L skim milk and 1.5g/L glycine). Samples were incubated with primary antibodies overnight at 4 °C. Subsequently, samples were incubated with 1:1000 dilutions of the anti-mouse IgG (H+L), F(ab')2 Fragment (Alexa Fluor® 488 Conjugate) or anti-rabbit IgG(H+L), F(ab') 2 Fragment (Alexa Fluor 488 conjugate) secondary antibodies. Nuclei were stained with DAPI (4,6-diamidino-2-phenylindole; Invitrogen). Images were detected using confocal electroscope.

MTT assay

Cells were seeded in 96-well plates and cultured at 37 °C with 5% CO_2 for 24, 48, 72 hrs, respectively. Then 10 mM 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Sigma) was added to cells and incubated for 4 hrs. Subsequently, medium was removed and 100 µl of DMSO was added to each well. The absorbance of each well was read on a microplate absorbance readers (BIO-RAD) at a wavelength of 490 nm. All measurements were performed in triplicate.

Statistical analysis

All results were presented as mean ± SEM. Comparisons between groups were performed with Mann-Whitney test. Differences were considered significant at a P value less than 0.05.

Results

A screening for kinases involved in antiviral immunity identifies conventional PKCs as novel cell-autonomous antiviral elements

Protein kinases are pivotal mediators of signal transduction and identifying kinases involved in biological responses can shed important light on kinase associated virus-host interactions. The lack of understanding as to which signal pathways mediate cell-autonomous antiviral immunity against HEV thus prompted us to execute a screening of kinase inhibitors with respect to their effects in antiviral responses. To this end, we employed a hepatocyte cell line, i.e. Huh7, transfected with a HEV replicon luciferase reporter as a platform for the screening of 132 pharmacological kinase inhibitors with known specificity profile [9] (Figure 1A and Table S1). We arbitrarily set the control luciferase value to 1 and identified 24 inhibitors that increase and 64 compounds that inhibit luciferase activity in this assay system (Figure 1B and Table S1). Inhibition of luciferase activity might be due to non-specific effects not related to the scientific question at hand (*e.g.* effects on translation or cell survival). Strikingly, stimulation of luciferase activity likely relates to the inhibition of signaling elements involved in constraining viral replication and hence we concentrated on luciferase enhancing compounds in our search for elements involved in antiviral immunity. The most clear and interesting of these enhancing compounds is Go6976, a fairly specific inhibitor of the conventional PKCs (PKCα, PKCβ_I, PKCβ_{II}, and PKCγ) [11]. Subsequent western blot analysis for the phosphorylation state of PKC isoforms confirmed the inhibition of PKCα and PKCβ by Go6976 in our experimental system (Figure 1C). HEV promoting activity of Go6976 was further confirmed in two independent state-of-the-art cell culture models: a subgenomic HEV containing luciferase reporter and a full-length HEV infectious cell culture system (Figure 1D and E).

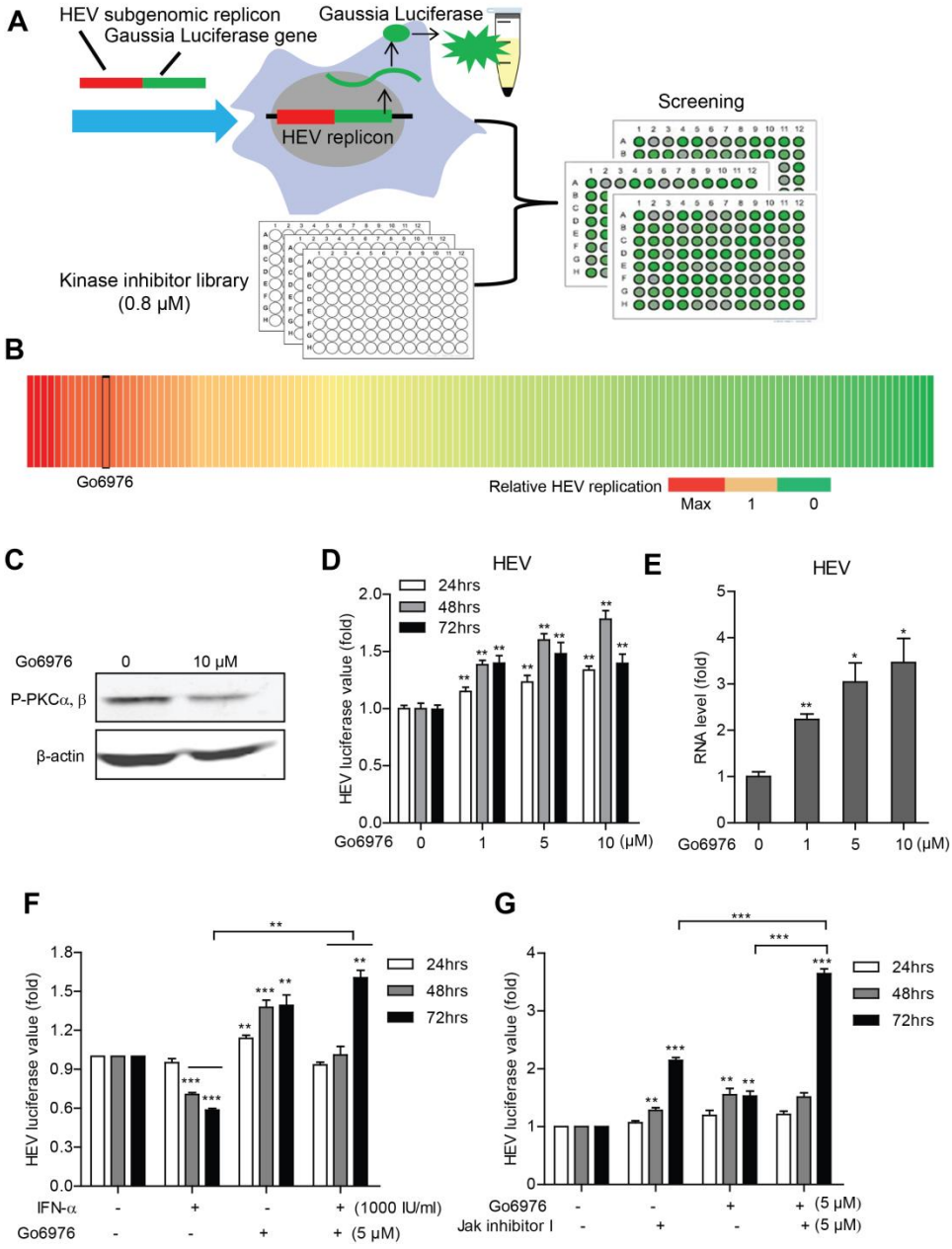

Figure 1. Conventional PKCs function as cell-autonomous antiviral elements against HEV. (A) Diagram of screening a library of pharmacological kinase inhibitors in HEV subgenomic replicon. (B) Heatmap summary of the screening results. The Huh7 cell line transfected with a subgenomic HEV luciferase reporter replicon was used. Compared to control, relative HEV luciferase activity depicted in red to green for each inhibitor. Red means that the signal is higher than control, whereas green means lower than control. See also Table S1. (C) Go6976 (10 μM) treatment inhibited phosphorylation of PKCα and PKCβ protein levels in Huh7 cells as determined by western blot. (D) In the Huh7 cell-based subgenomic HEV replicon model, treatment with different doses of Go6976 increased HEV replication-related luciferase activity (n = 3 independent experiments with 2 - 3 replicates each). (E) qRT-PCR analysis of HEV RNA in Huh7 cells harboring full-length HEV infectious genome. Treatment with Go6976 dose-dependently increased cellular HEV RNA (n = 3). (F) The Huh7 cell-based subgenomic HEV replicon, treated with GO6976, IFN-α or a combination of both. Luciferase values were measured at 24, 48 and 72 hours. IFN-α induced anti-HEV effect was totally abrogated with the present of Go6976 (n = 3 independent experiments with 2 – 3 replicates each). (G) Same as (F) for GO6976, *pan*-JAK inhibitor I or a combination of both. The co-treatment of Go6976 and *pan*-JAK inhibitor I exerted combined enhancement on HEV replication. (n = 3 independent experiments with 2 – 3 replicates each). Data presented as means ± SEM (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ns, not significant).

IFN-α is a well-known HEV off-label drug used in clinic. Its antiviral effect against HEV was totally abrogated with the present of Go6976 (Figure 1F). Whereas the co-treatment of Go6976 with *pan*-JAK inhibitor I, a well-established inhibitor used to block IFN signaling, exerted combined enhancement on HEV replication (Figure 1G). Go6976 showed a slight inhibitory effect on host cell growth (Figure S1A), which is expected because of the crucial roles of PKCs in cell physiology. Collectively, these data demonstrated that conventional PKCs are important antiviral elements, at least with respect to HEV infection.

PKCα is the key antiviral isoform against HEV

The observation that conventional PKCs constrain HEV replication raises questions as to the role of different PKC isoforms. To dissect the effects of individual PKC isoforms, we silenced the expression of *PRKCA* (the gene coding for PKCα) and *PRKCB* (that gives rise to $PKC\beta_I$ and $PKC\beta_{II}$) in Huh7 cells using lentiviral-mediated RNAi. Since PKCγ has been shown to be specifically expressed in neuronal tissue [12], we ruled it out for further research. qRT-PCR and Western blot confirmed successful down-regulation of PKC isoforms (Figure 2A and B) at protein and RNA levels. Subsequently, cells were inoculated with infectious HEV particles and cellular HEV RNA was quantified by qRT-PCR after 48 hrs. Knockdown of PKCα led to a 2.25 ± 0.3 fold ($n = 4$, $p < 0.05$) increase of HEV RNA; whereas PKCβ knockdown resulted in no significant effect (Figure 2C), suggesting that PKCα is the relevant isoform here.

PKCα maintains its inactive state via an inhibitory region within the effector binding domain of the kinase. Its pseudosubstrate site mediates this inhibition by binding to the active site and preventing substrate interaction [13]. A constitutively active PKCα (caPKCα) is available in which a glutamic acid present in this region is substituted for alanine. (Figure 2D). This form dramatically increases effector-independent kinase activity, compared to the wild-type PKCα (wtPKCα) [14]. Huh7 cells were transduced with integrating lentiviral vectors co-expressing GFP and caPKCα or wtPKCα (Figure S1B). Cell cytometry confirmed transgene expression by measuring GFP and positive cells were sorted and expanded for further experimentation (Figure S1C). Huh7 cells expressing caPKCα or wtPKCα were inoculated with infectious HEV particles and relative viral RNA level was quantified 48 hours post-inoculation. Consistent with PKCα knockdown (Figure 2C), expression of caPKCα significantly

decreased HEV RNA by 49% (n = 4, p < 0.05), while wtPKCα over-expression showed no effect on HEV compared to control sample (Figure 1E). Thus, activated PKCα plays an important role in cell-autonomous anti-HEV immunity.

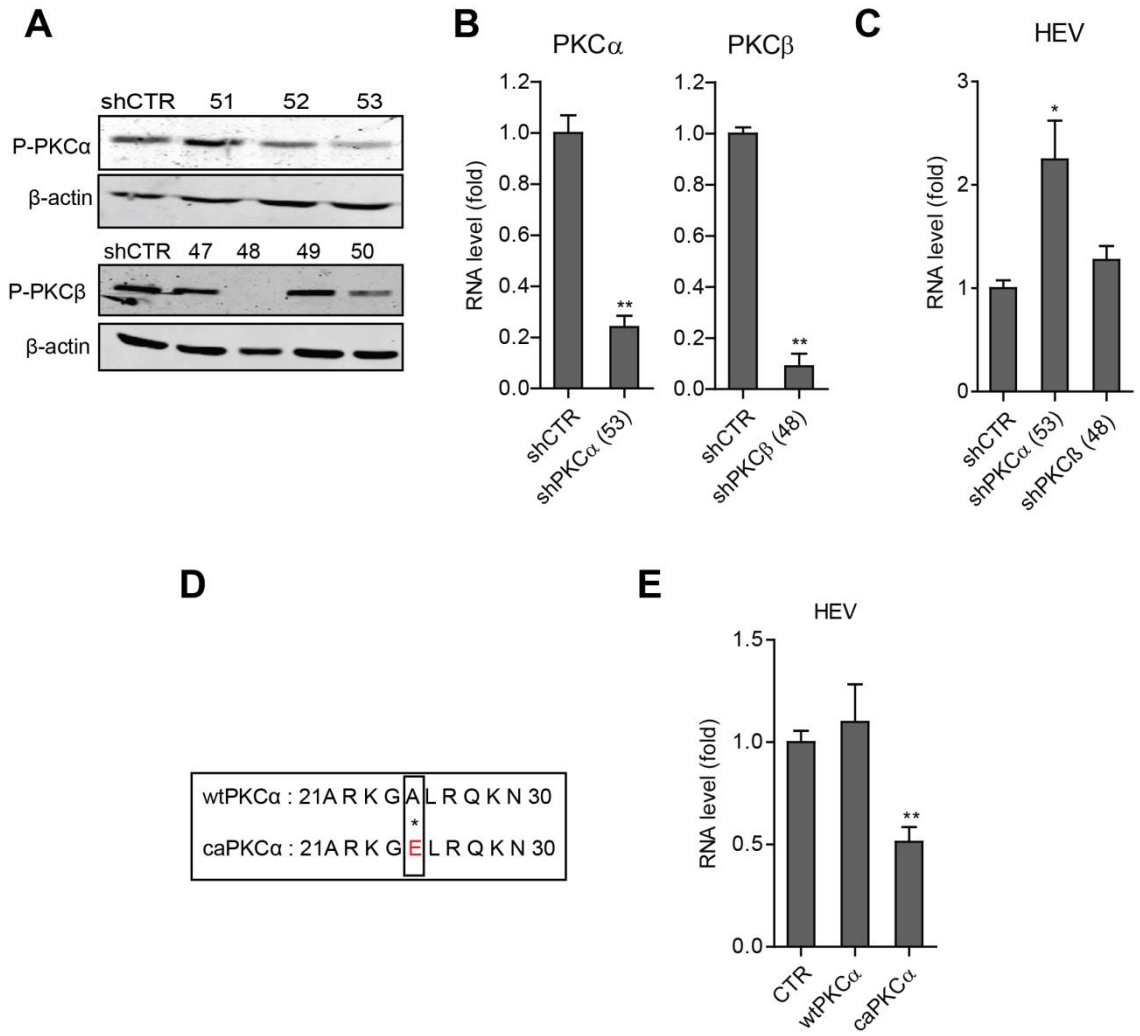

Figure 2. PKCα is the key antiviral isoform against HEV. (A) Western blot analysis of PKCα and PKCβ knockdown by lentiviral shRNA vectors. Compared with the control vector transduced cells, the shPKCα clone 53 and shPKCβ clone 48 exerts potent silencing capability shown at protein levels. Blots depict phosphorylated PKCα, PKCβ and β-actin. (B) qRT-PCR analysis of PKCα and PKCβ knockdown by lentiviral shRNA vectors. Compared to the control vector transduced cells, the no.53 and 48 clones of shPKCα and PKCβ, respectively, exert a potent silencing capability shown at RNA levels (n = 3). (C) Cellular HEV RNA level in PKCα or PKCβ knockdown cells was determined by qRT-PCR 48 hrs post inoculation with HEV particles. Knockdown of PKCα led to a 2.25 ± 0.3 fold increase of HEV RNA, whereas PKCβ knockdown resulted in no significant increase (n = 4). (D) The change in amino acid sequence between wtPKCα and caPKCα is shown in the rectangular frame. (E) qRT-PCR analysis of cellular HEV RNA level in CTR, wtPKCα or caPKCα over-expressing cells after inoculation of infectious HEV particles for 72 hrs. caPKCα over-expression inhibited HEV RNA by 49%. Data presented as mean ± SEM (*, P < 0.05; **, P < 0.01; ***, P < 0.001; ns, not significant).

This promising result prompts us to investigate the potential role of canonical PKC pharmacologic activator, phorbol 12-myristate 13-acetate (PMA), also commonly known as

12-*O*-Tetradecanoylphorbol-13-acetate (TPA). PMA, structurally analogous to diacylglycerol, is commonly used to activate PKC. It is also a promising drug candidate, currently under a Phase II clinical trial in the treatment of patients with relapsed/refractory acute myelogenous leukemia (NCT01009931). As expected, PMA exerted strong anti-HEV effects in both HEV subgenomic and full-length infectious models (Figure 3A and B), while no clear effect on cell growth and viability was observed (Figure S1D). This exciting result prompted us to assess the combined antiviral effect of PMA with the off-label anti-HEV drugs IFN-α or ribavirin. Although PMA and IFN-α showed comparable anti-HEV capacity, they failed to exert further combined effect (Figure 3C and E). Whereas the combination of PMA with ribavirin showed strong additive anti-HEV effect (Figure 3D and F). These data collectively indicate PMA as a potential anti-HEV drug candidates.

PKCα mediated anti-HEV effect is independent of NF-κB pathways

NF-κB signaling is a central pathway involved in cellular innate immune response. PMA can activate NF-κB signaling via the phosphorylation of NF-κB/p65 by PKCα (Figure 4A) [15-17]. Thus, we investigated the potential involvement of NF-κB pathway in PKCα mediated anti-HEV effect. To this end, we used a lentiviral transcriptional reporter system expressing the firefly luciferase gene under the control of NF-κB responsive promoter. Huh7 cells were transduced with the vector to create a stable NF-κB reporter cell line. As expected, stimulation with PMA led to the strong activation of NF-κB luciferase activity and thus a role of NF-κB signaling cannot be ruled out. Thus, the Huh7 cell line was transduced with integrating lentiviral shRNA vectors to silence RelA (P65), an essential subunit of the NF-κB transcription complex, resulting in profound down-regulation of RelA expression (Figure 5C and D). However, PMA induced anti-HEV effect was not abrogated in RelA knockdown cells (Figure 5E). Thus, NF-κB signaling appears not to be involved in the PKCα-induced anti-HEV effect.

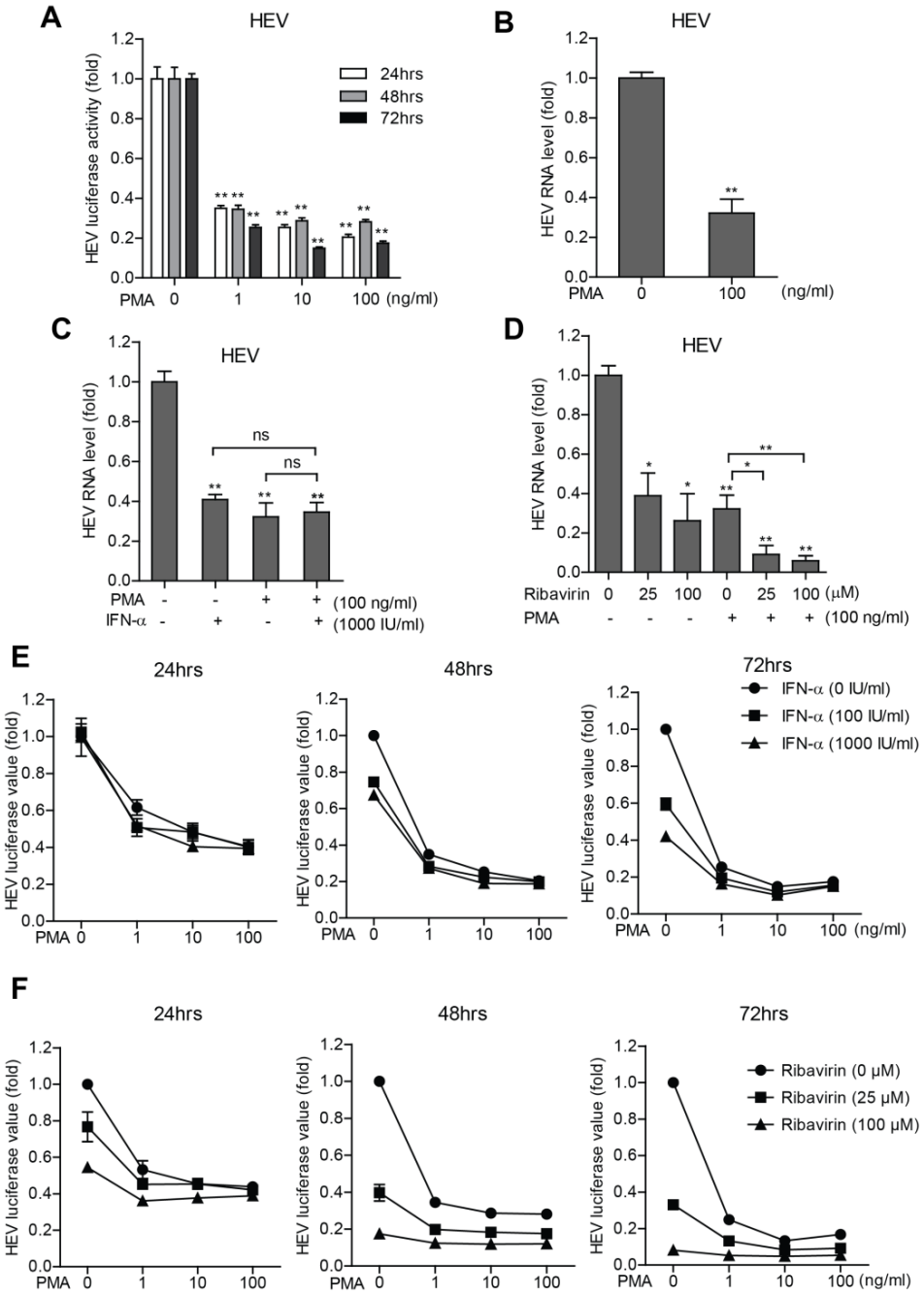

Figure 3. PKC specific activator PMA exerts strong antiviral activity against HEV. (A) In the Huh7 cell-based subgenomic HEV replicon model, treatment with different doses of PMA inhibited HEV replication-related luciferase activity (n = 3 independent experiments with2 - 3 replicates each). (B) qRT-PCR analysis of HEV RNA derived from Huh7 cells harboring the full-length HEV infectious genome. Treatment with PMA (100 ng/ml) for 48 hrs significantly inhibited cellular HEV RNA by 68% (n = 9). (C) Huh7 cells harboring the full-length HEV infectious genome were treated with different doses of IFN-α, PMA or a combination of both for 48 hrs. Cellular HEV RNA level was determined by qRT-PCR. PMA and IFN-α showed comparable anti-HEV capacity, but they failed to exert further combined effect (n = 4). (D) Same as (C) for ribavirin, PMA or a combination of both. The combination of PMA with ribavirin showed strong additive anti-HEV effect (n = 4). (E) The Huh7 cell-based subgenomic HEV replicon was treated with PMA, IFN-α or a combination of both. Their combination showed no additive effect. Luciferase values were measured at 24, 48 and 72 hrs. (n = 3 independent experiments with 2 – 3 replicates each). (F) Same as (E) for PMA, ribavirin or a combination of both. Their combination showed additive anti-HEV activity. (n = 3 independent experiments with 2 – 3 replicates each). Data presented as means ± SEM (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ns, not significant).

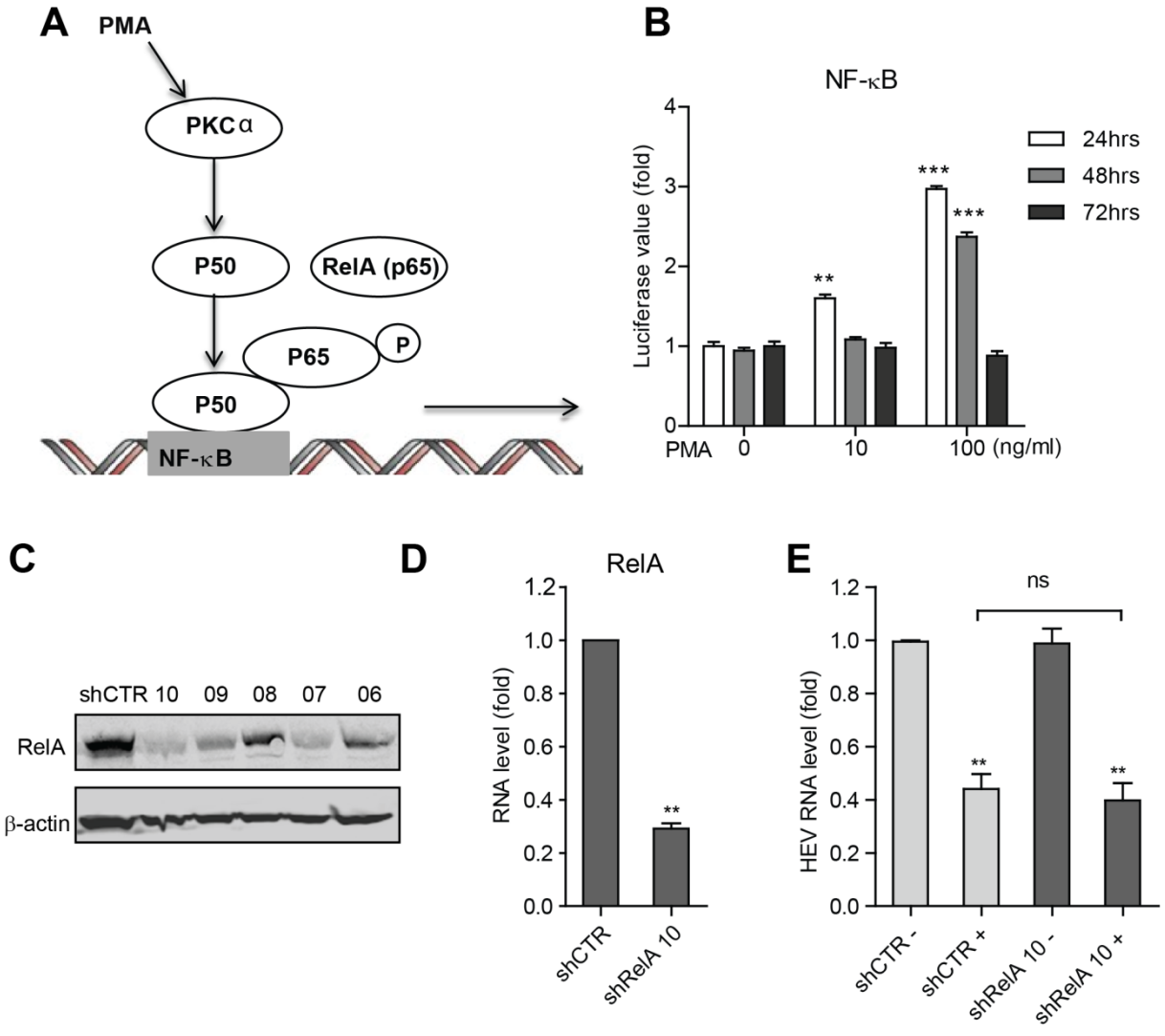

Fig 4. PKCα mediated anti-HEV activity is independent of NF-κB signaling. (A) Illustration of key regulatory molecules in PMA induced NF-κB signaling. (B) In Huh7 based NF- κB luciferase reporter cells, treatment with PMA resulted in a dose-dependent induction of NF-κB related luciferase activity (n = 3 independent experiments with 2 – 3 replicates each). (C) Western blot analysis of RelA knockdown by lentiviral shRNA vectors in Huh7 cells. Blots depict RelA and β-actin. (D) qRT-PCR analysis of RelA knockdown by lentiviral shRNA vectors at RNA level (n = 3). (E) Knockdown of RelA did not block PMA induced anti-HEV activity as determined by qRT-PCR 48 hrs post inoculation with HEV particles (n = 4). Data presented as mean ± SEM (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ns, not significant).

PKCα mediated anti-HEV effect is independent of AP-1 pathways

AP-1 signaling is another important pathway involved in cellular innate immune response. PMA can also activate AP1 signaling via the activation of PKCα (Figure 5A) [18,19]. Indeed, PMA stimulation provokes a strong induction of c-Fos, an essential subunit of the AP1 transcription complex (Figure 5B). Accordingly, unstimulated cells displayed hardly

detectable c-Fos protein, but c-Fos was substantially induced and translocated to nucleus following PMA stimulation (Figure 6C). Convincingly, we also used a lentiviral transcriptional reporter system expressing the firefly luciferase gene under control of an AP-1 responsive promoter. Huh7 cells were transduced with the vector to create a stable AP-1 reporter cell line. As shown in Figure 5D, stimulation with PMA led to the strong activation of AP-1 luciferase activity. Thus, to determine the role of AP-1 activation, Huh7 cells were transduced with integrating lentiviral RNAi vectors to silence *c*-Fos (Figure 5E and F). Surprisingly, PMA mediated anti-HEV activity was not changed in c-Fos knockdown cells when compared with control cells (Figure 5G). Thus, AP-1 appears not essential for PKCα-mediated anti-HEV activity.

Discussion

Protein kinases play pivotal roles in regulating immune responses either positively or negatively via regulating protein functions, signal transduction or other cellular processes.[20-22]. This study comprehensively profiled kinase-mediated cascades in cell-autonomous antiviral immunity via screening a library of pharmacological kinase inhibitors on Huh7 based HEV replication cell model. We identified PKCα as an important antiviral host factor and a targetable host factor for antiviral drug development. PKCα has been reported to be involved in several cellular innate immune responses, such as enhancing NF-κB translocation, participating in Toll like receptor signaling and modulating IFN-β synthesis in particular cell types. In this study, we identified PKCα as a powerful antiviral effector against HEV infection. Both functional over-expression and pharmacologic activation showed strong and comparable anti-HEV activity compared to HEV off-label drug IFN-α.

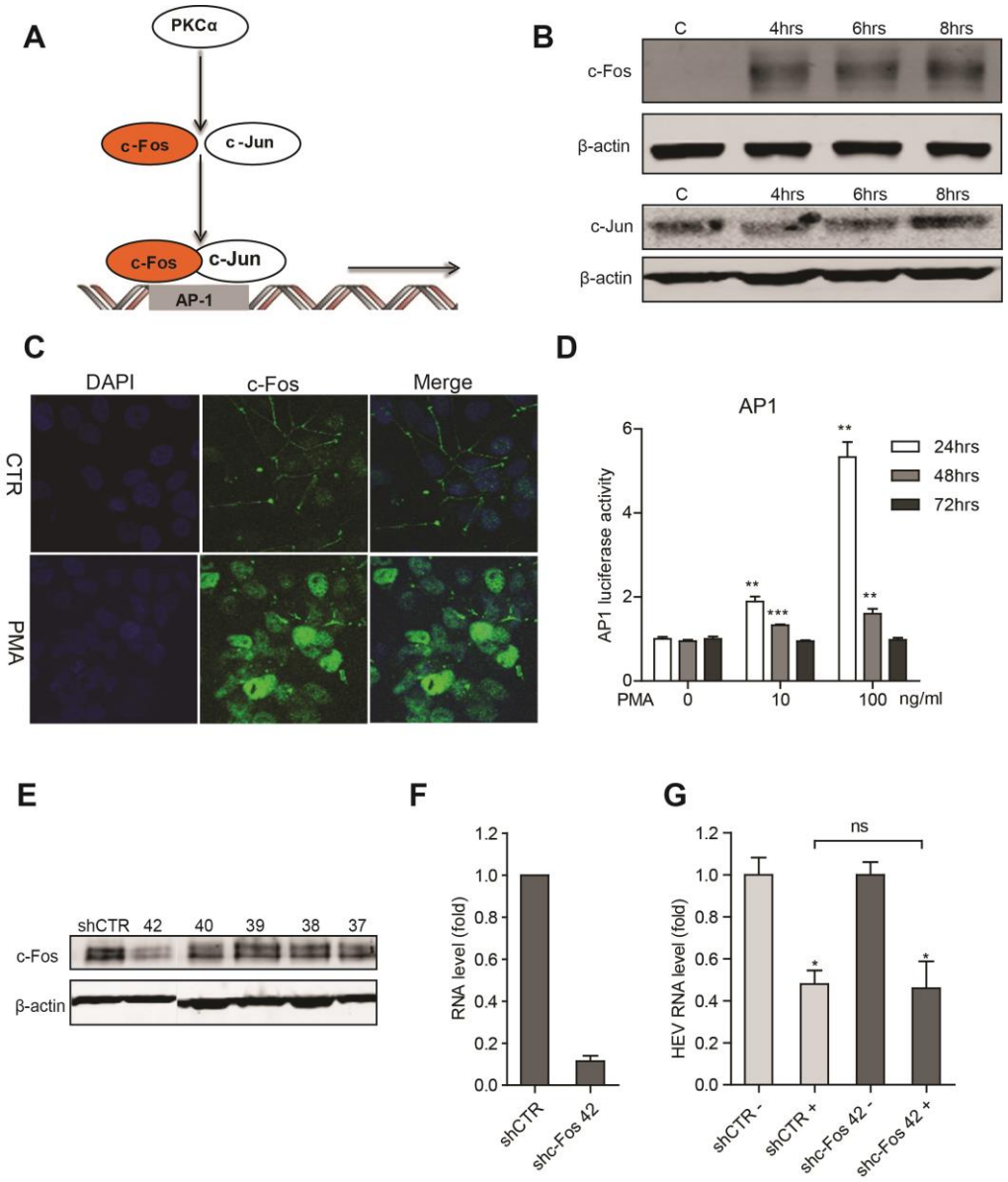

Figure 5. PKCα mediated anti-HEV activity is independent of AP-1 signaling (A) Diagram of key regulatory molecules in the PKC/AP-1 signaling pathway. (B) Western blot analysis of c-Fos and c-Jun protein levels in Huh7 cells treated with PMA (100 ng/ml) for 4, 6 and 8 hrs. PMA stimulation provokes strong induction of c-Fos but not c-Jun. (C) Confocal microscopy analysis of c-Fos localization in Huh7 cells treated with PMA for 4 hrs. c-Fos was induced and translocated to nucleus upon PMA stimulation. c-Fos antibody (green). Nuclei were visualized by DAPI (blue). (D) In Huh7 based AP-1 luciferase reporter cells, treatment with PMA resulted in dose-dependent induction of AP-1-related luciferase activity (n = 3 independent experiments with 2 – 3 replicates each). (E) Western blot analysis of c-Fos knockdown by lentiviral shRNA vectors. Compared to the control vector transduced cells, the NO.42 clone of shc-Fos exerts potent silencing capability shown at protein levels. Blots depict c-Fos and β-actin. (F) qRT-PCR analysis of c-Fos knockdown by lentiviral shRNA vectors at RNA level (n = 3). (G) Knockdown of c-Fos in Huh7 cells did not abrogate PMA induced anti-HEV activity as determined by qRT-PCR 48 hrs post inoculation with HEV particles (n = 4). Data presented as means ± SEM (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ns, not significant).

PMA, as a phorbol ester, binds to the C1 domain in the regulatory region of PKCs to promote their activation [23]. Although PMA was reported to may have a tumor-promoting

role in experimental skin cancer mouse models [24], its anti-cancer potential in fact has been extensively investigated in the clinic, including in patients with hematological malignancy, squamous cell carcinoma, renal cell carcinoma, ovarian teratocarcinoma, subcutaneous adenocarcinoma and prostate cancer [25]. Cancer patients often suffer from depressed white blood cell and neutrophil counts because of chemotherapeutic drugs. PMA treatment has been shown to increase white blood cell and neutrophil counts towards a normal range with only mild and reversible side effects observed [26]. A Phase I trial of treating hematologic cancer or bone marrow disorder with PMA has been successfully conducted at The State University of New Jersey (NCT00004058). The same institute is currently pursuing a Phase II trial plus dexamethasone & choline magnesium trisalicylate in the treatment of patients with relapsed/refractory acute myelogenous leukemia (NCT01009931). An interesting link is that patients with leukemia or other cancers are prone to virus infections, including HEV [27-29] The potential clinical prospects of PMA or its derivatives may be of achieving “one stone two birds” effects: simultaneously combating cancer and virus.

In conclusion, we identified PKCα as an important cell-autonomous antiviral factor against HEV in host defense. The anti-HEV activity is totally independent of its downstream NF-κB or AP-1 pathways These results provide valuable antiviral target and shed new insights of virus-host interactions.

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Supplementary Materials

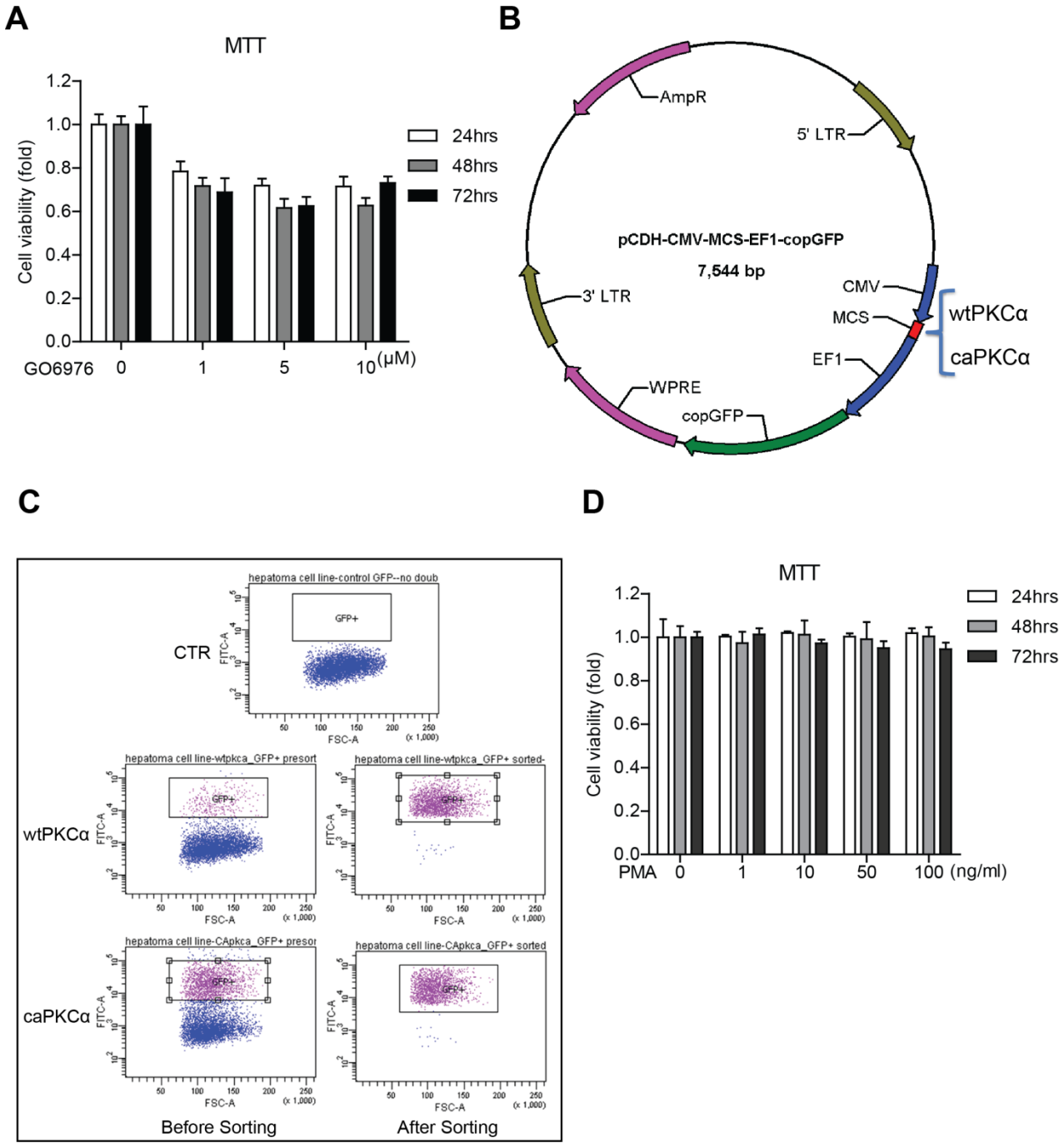

Figure S1. PKCα exerts strong anti-HEV activity. (A) MTT assay to determine GO6976 effects on the Huh7 cell line. Go6976 showed a slight inhibitory effect. (B) Illustration of lentivector map of wtPKCα and caPKCα. (C) Cell cytometry sorting result of wtPKCα and caPKCα positive cells based on the GFP tag. (D) MTT assay to determine PMA effects on the Huh7 cell line. PMA exerts no clear cell toxicity

Table S1. the library information of pharmacological kinase inhibitors

Name	Formula	MolWeight	Catalog Number
Alsterpaullone, 2-Cyanoethyl	C19H14N4O3	346,33946	12687 1
Cdc2-Like Kinase Inhibitor, TG003	C13H15NO2S	249,3287	219479
Cdk1 Inhibitor	C17H11ClN2O	294,73504	217695
Aurora Kinase/Cdk Inhibitor	C17H15F2N7O3S	435,4079064	189406
Cdk4 Inhibitor II, 625987	C15H13NO2S	271,33422	219477
GSK-3 Inhibitor IX	C16H10BrN3O2	356,1735	361550
MK2a Inhibitor	C22H20FNO2	349,3981032	475863
GSK-3 Inhibitor X	C18H12BrN3O3	398,21018	361551
DNA-PK Inhibitor III	C12H15NO3	221,2524	260962
ERK Inhibitor II, Negative Control	C18H12N6O	328,32748	328008
Gö 6983	C26H26N4O3	442,50964	365251
Gö 6976	C24H18N4O	378,42592	365250
GSK-3b Inhibitor VIII	C12H12N4O4S	308,31308	361549
AG 1024	C14H13BrN2O	305,16982	121767
Indirubin-3'-monoxime	C16H11N3O2	277,27744	402085
Aloisine, RP106	C17H19N3O	281,35226	128135
EGFR/ErbB-2/ErbB-4 Inhibitor	C17H11ClFN5O	355,7535432	324840
PKR Inhibitor	C13H8N4OS	268,29382	527450
BAY 11-7082	C10H9NO2S	207,24896	196870
GTP-14564	C15H10N2O	234,2527	371806
PKCb Inhibitor	C24H21N5O2	411,45584	539654
VEGF Receptor	C19H16ClN3O	337,80284	676481

Tyrosine Kinase Inhibitor II			
Bohemine	C18H24N6O	340,42276	203600
Cdk1/2 Inhibitor III	C15H13F2N7O2S2	425,4362264	217714
VEGF Receptor 2 Kinase Inhibitor IV	C17H13N3OS	307,36962	676489
JAK3 Inhibitor II	C16H14BrN3O3	376,20466	420104
2826-26-8	C17H14N2O3	294,30466	658401
PD 158780	C14H12BrN5	330,18258	513035
PD 174265	C17H15BrN4O	371,2312	513040
Casein Kinase II Inhibitor III, TBCA	C9H4Br4O2	463,74286	218710
VEGF Receptor 2 Kinase Inhibitor III	C15H14N2O	238,28446	676487
Cdk2 Inhibitor IV, NU6140	C23H30N6O2	422,5233	238804
IRAK-1/4 Inhibitor	C20H21N5O4	395,41184	407601
Indirubin Derivative E804	C20H19N3O4	365,38256	402081
Flt-3 Inhibitor II	C17H12N2O3	292,28878	343021
Aurora Kinase Inhibitor III	C21H18F3N5O	413,3957296	189405
LY 294002	C19H17NO3	307,34318	440202
Cdk4 Inhibitor III	C15H12N2O2S	284,33298	219478
ERK Inhibitor II, FR180204	C18H13N7	327,34272	328007
AGL 2043	C15H12N4S	280,34758	121790
PKCbII/EGFR Inhibitor	C20H13F2N3O2	365,3329264	539652
Diacylglycerol Kinase Inhibitor II	C28H25F2N3OS	489,5794064	266788
Chelerythrine	C21H18ClNO4	383,82492	220285

Chloride			
DNA-PK Inhibitor V	C17H17NO3	283,32178	260964
Chk2 Inhibitor II	C20H14ClN3O2	363,79706	220486
JAK Inhibitor I	C18H16FN3O	309,3375432	420099
PP1 Analog II, 1NM-PP1	C20H21N5	331,41424	529581
VEGF Receptor Tyrosine Kinase Inhibitor III, KRN63	C20H21ClN4O4	416,85814	676482
Rho Kinase Inhibitor III, Rockout	C13H10N2	194,2319	555553
GSK-3b Inhibitor II	C14H10IN3OS	395,21817	361541
AG 1295	C16H14N2	234,29576	658550
GSK-3 Inhibitor XIII	C18H15N5	301,3452	361555
ROCK Inhibitor, Y-27632ROCK Inhibitor, Y-27632	C14H25Cl2N3O2	338,2732	688000
Isogranulatimide	C15H8N4O2	276,24962	371957
Cdk2 Inhibitor III	C20H28N6O3	400,47472	238803
LY 303511	C19H18N2O2	306,35842	440203
AG 9	C11H8N2O	184,19402	658390
PI 3-KbInhibitor II	C11H5F2NO4S	285,2235064	528108
Compound 56Compound 56	C18H18BrN3O2	388,25842	234505
PKR Inhibitor, Negative Control	C15H8Cl3NO2	340,58852	527455
VEGF Receptor 2 Kinase Inhibitor II	C17H15BrN2O	343,2178	676485
AG 112	C13H8N4O	236,22882	658440
Flt-3 Inhibitor III	C21H23N3OS	365,49182	343022
Syk Inhibitor II	C14H21Cl2F3N6O3	449,2561496	574712

PI-103	C19H16N4O3	348,35534	528100
Syk Inhibitor	C18H15N3O3S	353,395	574711
Alsterpaullone	C16H11N3O3	293,27684	126870
IC261	C18H17NO4	311,33188	400090
ATM Kinase Inhibitor	C21H17NO3S2	395,49458	118500
AG 1478	C16H14ClN3O2	315,75426	658552
JNK Inhibitor V	C20H16N6S	372,44624	420129
EGFR/ErbB-2 Inhibitor	C23H21N3O3	387,43114	324673
Flt-3 Inhibitor	C18H20N2O4S	360,4274	343020
JAK3 Inhibitor IV	C23H26ClNO	367,91164	420121
SU6656	C19H21N3O3S	371,45334	572635
DNA-PK Inhibitor II	C17H15NO3	281,3059	260961
cFMS Receptor Tyrosine Kinase Inhibitor	C20H22N4O3	366,41368	344036
VEGF Receptor 2 Kinase Inhibitor I	C18H18N2O3	310,34712	676480
Aminopurvalanol A	C19H26ClN7O	403,90904	164640
Compound 52	C16H19ClN6O	346,81466	234503
TGF-b RI Kinase Inhibitor	C17H12N4	272,30398	616451
AMPK Inhibitor, Compound C	C24H25N5O	399,4882	171260
Cdk1/5 Inhibitor	C9H7N5	185,18538	217720
Casein Kinase I Inhibitor, D4476	C23H18N4O3	398,41402	218696
TGF-b RI Inhibitor III	C20H22ClN3O2	371,86058	616453
Syk Inhibitor III	C9H7NO4	193,15618	574713
Cdk4 Inhibitor	C20H10BrN3O2	404,2163	219476
PDGF Receptor	C27H27N5O4	485,53438	521232

Tyrosine Kinase Inhibitor III			
Bcr-abl Inhibitor	C18H13F3N4O2	374,3166296	197221
Rho Kinase Inhibitor IV	C18H28Cl2N4O4S	467,41032	555554
Akt Inhibitor V, Triciribine	C13H16N6O4	320,30394	124012
BPIQ-I	C16H12BrN5	354,20398	203696
ERK Inhibitor III	C12H10N6O5	318,245	328009
PI 3-Kg Inhibitor	C12H7N3O2S	257,26788	528106
GSK-3b Inhibitor XI	C18H15N5O3	349,3434	361553
JNK Inhibitor II	C14H8N2O	220,22612	420119
Aurora Kinase Inhibitor II	C23H20N4O3	400,4299	189404
JNK Inhibitor IX	C20H18N2O2S	350,43412	420136
GSK-3b Inhibitor I	C10H10N2O2S	222,2636	361540
IKK-2 Inhibitor IV	C12H10FN3O2S	279,2901032	401481
PP3	C11H9N5	211,22266	529574
Lck Inhibitor	C23H22N4O	370,44698	428205
Src Kinase Inhibitor I	C22H19N3O3	373,40456	567805
Aloisine A, RP107	C16H17N3O	267,32568	128125
AG 1296	C16H14N2O2	266,29456	658551
IGF-1R Inhibitor II	C18H16ClN3O2	341,79154	407248
ATM/ATR Kinase Inhibitor	C23H18Cl3FN4O3S	555,8364232	118501
Bisindolylmaleimide IV	C20H13N3O2	327,33612	203297
JAK3 Inhibitor VI	C19H17N3O4S	383,42098	420126
Staurosporine, N-benzoyl-	C35H30N4O4	570,6371	539648
Akt Inhibitor X	C20H26Cl2N2O	381,33924	124020

JNK Inhibitor VIII	C18H20N4O4	356,3758	420135
PDGF Receptor Tyrosine Kinase Inhibitor II	C21H18N2O3	346,37922	521231
PDGF Receptor Tyrosine Kinase Inhibitor IV	C18H16FN3O2	325,3369432	521233
PDGF RTK Inhibitor	C26H29N3O7S	527,58936	521234
JNK Inhibitor, Negative Control	C15H10N2O	234,2527	420123
Aurora Kinase Inhibitor III	C21H18F3N5O	413,3957296	189405
Met Kinase Inhibitor	C28H30ClN5O4S	568,0869	448101
Bisindolylmaleimide I	C25H24N4O2	412,48366	203290
Cdk/Crk Inhibitor	C23H22Cl2N4O3	473,35178	219491
DMBI	C17H16N2O	264,32174	317200
EGFR Inhibitor	C21H18F3N5O	413,3957296	324674
KN-62	C38H35N5O6S2	721,8444	422706
Cdk1 Inhibitor, CGP74514A	C19H24ClN7	385,89376	217696
SU11652	C22H27ClN4O2	414,92838	572660
Rapamycin	C51H79NO13	914,17186	553210
Akt Inhibitor VIII, Isozyme-Selective, Akti-1/2	C34H29N7O	551,64036	124018
PDK1/Akt/Flt Dual Pathway Inhibitor	C10H4N6O	224,17836	521275
Fascaplysin, synthetic	C18H11ClN2O	306,74574	341251
Herbimycin A, Streptomyces sp.	C30H42N2O9	574,66248	375670
Staurosporine,	C28H26N4O3	466,53104	569397

Streptomyces sp.			
Akt Inhibitor IV	C31H27IN4S	614,54235	124011

Table S2. shRNA sequences

Name	Oligo Sequences(5' - 3')
shPKCα-51	CCGGCTTTGGAGTTTCGGAGCTGATCTCGAGATCAGCTCCGAAACTCCAAAGTTTTT
shPKCα-52	CCGGCGAGCTATTTCAGTCTATCATCTCGAGATGATAGACTGAAATAGCTCGTTTTT
shPKCα-53	CCGGCATGGAACTCAGGCAGAAATTCTCGAGAATTTCTGCCTGAGTTCCATGTTTTT
shPKCβ-47	CCGGCGTCCTTCATTTCTGTCATTCCTCGAGGAATGACAGAAATGAAGGACGTTTTTT G
shPKCβ-48	CCGGGCCATGAATTTGTCACATTCTCTCGAGAGAATGTGACAAATTCATGGCTTTTTTG
shPKCβ-49	CCGGGAAACAAAGATGGTTGTATTCCTCGAGGAATACAACCATCTTTGTTTCTTTTTTG
shPKCβ-50	CCGGGACGACCTGCTTTGATTTAACCTCGAGGTTAAATCAAAGCAGGTCGTCTTTTTT G
shRelA-06	CCGGGCCTTAATAGTAGGGTAAGTTCTCGAGAACTTACCCTACTATTAAGGCTTTTT
shRelA-07	CCGGCGGATTGAGGAGAAACGTAAACTCGAGTTTACGTTTCTCCTCAATCCGTTTTT
shRelA-08	CCGGGCAGGCTATCAGTCAGCGCATCTCGAGATGCGCTGACTGATAGCCTGCTTTTT
shRelA-09	CCGGCACCATCAACTATGATGAGTTCTCGAGAACTCATCATAGTTGATGGTGTTTTT
shRelA-10	CCGGCCTGAGGCTATAACTCGCCTACTCGAGTAGGCGAGTTATAGCCTCAGGTTTTT
shc-Fos-37	CCGGGCTGGTAGTTAGTAGCATGTTCTCGAGAACATGCTACTAACTACCAGCTTTTT
shc-Fos-38	CCGGGCGGAGACAGACCAACTAGAACTCGAGTTCTAGTTGGTCTGTCTCCGCTTTTT
shc-Fos-39	CCGGCACTGCTTACACGTCTTCCTTCTCGAGAAGGAAGACGTGTAAGCAGTGTTTTT
shc-Fos-40	CCGGGTGGAACAGTTATCTCCAGAACTCGAGTTCTGGAGATAACTGTTCCACTTTTT
shc-Fos-42	CCGGGCGGAGACAGACCAACTAGAACTCGAGTTCTAGTTGGTCTGTCTCCGCTTTTTG

Table S3. Primer sequences

Gene	Sequences 5’ to 3’
HEV-F	5’-ATTGGCCAGAAGTTGGTTTTCAC-3’
HEV-R	5’-CCGTGGCTATAATTGTGGTCT-3’
PKCα-F	5'-GCCTATGGCGTCCTGTTGTATG-3'
PKCα-R	5'-GAAACAGCCTCCTTGGACAAGG-3'
PKCβ-F	5'-GAGGGACACATCAAGATTGCCG-3'
PKCβ-R	5'-CACCAATCCACGGACTTCCCAT-3'
GAPDH-F	5’-TGTCCCCACCCCCAATGTATC-3’
GAPDH-R	5’-CTCCGATGCCTGCTTCACTACCTT-3’
RelA-F	5’-TGAACCGAAACTCTGGCAGCTG-3’
RelA-R	5’-CATCAGCTTGCGAAAAGGAGCC-3’
c-Fos-F	5’-GCCTCTCTTACTACCACTCACC-3’
c-Fos-R	5’-AGATGGCAGTGACCGTGGGAAT-3’

CHAPTER 8

Hepatitis E virus Infection Induces Mitochondrial Fusion to Facilitate Viral Replication

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Abstract

The liver is rich in mitochondria, an organelle which plays important roles in physiology and pathogenic processes. Viruses have been reported to manipulate mitochondrial dynamics, especially their fusion and fission to facilitate their infection. This study aims to investigate whether hepatitis E virus (HEV) alters mitochondrial dynamics and how these effects relate to the infectious process. We observe that HEV promotes mitochondrial fusion, as evident from a dramatic increase in mitochondrion length following infection. Mechanistically, these effects relate to HEV-dependent increased expression of OPA1 and Mfn1, which are both established mediators of mitochondrial fusion. Consistently, in OPA1 and Mfn1 knock-down cells, HEV no longer provoked mitochondrial fusion and this effect coincided with a significant reduction in HEV RNA levels, suggesting that HEV-induced mitochondrial fusion facilitates viral infection. Since mitochondrial dynamics have been reported to be strongly associated with innate immunity, we therefore explored the effect of inhibiting mitochondrial fusion on the cell-autonomous anti-viral immune response. Interestingly, production of the antiviral interferon-stimulated genes (ISGs) was substantially enhanced in OPA1- or Mfn1-knock-down cells. In conjunction our data suggest that HEV-induced mitochondrial fusion facilitates hepatocyte HEV infection by interfering with cell-autonomous antiviral immunity.

Introduction

Hepatitis E virus (HEV) infection is the most common cause of acute viral hepatitis world-wide. HEV is a single-stranded positive-sense RNA virus, which mainly infects liver hepatocytes. In general, HEV infection is a self-limiting disease and associated with low mortality, however, more than 60% of organ recipients infected with HEV will develop chronic hepatitis with rapid progression to cirrhosis [1]. Despite HEV infection being an emerging global health issue, no effective anti-HEV therapy is currently available. Only type I interferons (IFN-α/β), ribavirin or a combination as off-labelled compounds for clinically treatment are available, thus there is an urgent for novel approaches to combat infection [2]. The lack of knowledge as to the interaction of HEV with the hepatocellular host cell machinery represents a major gap in our understanding of the biology of this virus and hampers design of rational treatment.

Viral life cycles intimately interact with all aspect of cellular metabolism and accordingly also interact with host cell organelles. For survival and replication, viruses corrupt normal cell organelle function to interfere with anti-viral immunity. In this respect especially mitochondria appear interesting as there is substantial evidence that various viruses modulate mitochondrial behavior to facilitate infection [3]. The exact mechanisms employed by viruses to affect mitochondria are still very much subject to an intense research effort but especially mitochondrial dynamics have been proposed to constitute a principal target in this respect [4]. Mitochondrial dynamics involve continuous fusion of these organelles (leading to more elongated mitochondria) and their fission (division of mitochondria, leading to smaller organelles). These processes are subject to tight regulation and can be adapted as to provide adequate responses to alterations in the cellular environment, such as stress, infection, or inhibition of ATP production [5]. The balance between mitochondrial fusion and fission is constantly adapted as to allow optimal maintenance of mitochondrial integrity and energy supply, but also plays roles in metabolite maintenance, apoptosis, autophagy and viral elimination [6, 7]. For many viruses, however, the interaction with mitochondrial dynamics remains unclear and the importance of such events for infection uncharacterized. Also as to how hepatocyte HEV infection influences mitochondrial dynamics has not yet been investigated.

Earlier dysfunctional mitochondrial dynamics have been strongly linked to neurodegenerative disease. Knowledge on the role of mitochondrial dynamics in viral infection is still immature and scanty, but might provide a novel avenue in explaining virus-host interaction. Indeed, several viruses have been reported to manipulate the mitochondrial dynamics of host cells, influencing the balance between fusion and fission to facilitate their infections [8-11]. Nevertheless, the role of altered mitochondrial dynamics remains underexplored.

Therefore, in this study we investigated the role of mitochondrial dynamics and its regulatory factors in HEV replication, as well as the potential involvement of altered dynamics in the anti-viral immune response. We found that HEV infection provokes profound modulation of mitochondrial dynamics through regulation of OPA1 and Mfn1 and that this effect is important in the HEV life cycle by impairing cell- autonomous immunity against HEV.

Materials and Methods

Compounds

Ethidium bromide (EB) (CAS 1239-45-8) was bought from Sigma, 2',3'-Dideoxycytidine (ddC) was bought from ITK Diagnostics B.V. (CAS 7481-89-2). goat anti-HSP60 (N-20) was bought from Bio-connect. Alexa Fluor® 488 AffiniPure Donkey Anti-Goat IgG (H+L) secondary Antibody was from Jackson ImmunoResearch. Goat anti-Mouse IgG (H+L) Secondary Antibody, Alexa Fluor® 555 conjugate was from Invitrogen. OPA1 antibody was brought from BD pharmingen.

Cell Cultures

Human hepatoma cell line huh7 and human embryonic kidney epithelial cell line 293T were cultured in Dulbecco's modified Eagle medium (DMEM) (Invitrogen) supplemented with 10% fetal bovine serum, 100 IU/ml penicillin, and 100 IU/ml streptomycin.

Cell culture models

An HEV replication model with subgenomic HEV sequence coupled with a Gaussia luciferase reporter gene and an HEV infection model containing the full-length HEV genome

were used in our study. HEV genomic RNA was generated from a plasmid construct containing the full-length HEV genome (Kernow-C1 p6 clone, GenBank Accession Number JQ679013) or a construct containing subgenomic HEV sequence coupled with a Gaussia luciferase reporter gene (p6-luc), using the Ambion MESSAGE MACHINE *in vitro* RNA transcription Kit (Life Technologies Corporation). All cells were grown at 37 °C, 5% CO2, and 100% humidity. Huh7 cells constitutively expressing the firefly luciferase reporter gene driven by the human PGK promoter were used to represent household luciferase activity for normalizing nonspecific effects on luciferase activity.

Quantification of Hepatitis E Virus Infection

RNA was isolated with a Machery-Nucleo Spin RNA II kit (Bioke, Leiden, The Netherlands) and quantified using a Nanodrop ND-1000 (Wilmington, DE, USA). cDNA was prepared from total RNA using a cDNA Synthesis Kit (TAKARA BIO INC). The cDNA was quantified with a SYBR Green-based real-time PCR (MJ Research Opticon, Hercules, CA, USA) according to the manufacturer's instructions. GAPDH or β-actin was considered as reference gene to normalize gene expression.

Immunofluorescence and Confocal microscope

For immunofluorescence staining, cells grown on glass cover slips were fixed with 4% formaldehyde in phosphate-buffered saline solution (PBS) for 15 min at room temperature. Slips were permeabilized with 0.1% Triton X-100 in phosphate-buffered saline (PBS) for 10 min and then blocked by % H2O2 in PBS for 5min. The slips were incubated with primary antibody anti-HSP60 overnight at 4°C, washed with PBS for 3 times, and incubated with secondary antibodies for 1 h at room temperature. After 3 times washing using PBS, DAPI staining was applied (nuclei). Images were observed under a Zeiss LSM 510 confocal microscope with a Plano Apochromat 63×/1.4-numerical-aperture oil immersion objective. Confocal images were acquired using Zeiss ZEN 2010LSM software.

Electroporation of HEV RNA

Huh-7 cells were collected and centrifuged for 5 min, 1500 rpm, 4°C. The supernatant was removed and the cells were washed with 4 ml Opti-DMEM (Thermo Scientific, the Neterlands) by centrifuging for 5 min, 1500 rpm, 4°C. The cell pellet was re-suspended in

100 µl Opti-DMEM and mixed with p6 full-length HEV RNA or p6-Luc subgenomic RNA. Electroporation was performed with the Bio-Rad's electroporation systems using the protocol of a designed program (400 volt, pulse length 0.5, number 1 and cuvette 4 mm).

Mitochondrial length quantification

Mitochondrial length was assessed by staining with HSP60 antibody and measured by tracing the mitochondria using ImageJ software. Mitochondrial length was binned into different categories (<0.5 mm, 0.5–1 mm, 1–2 mm, and >2 mm).

Gene knockdown by lentiviral vector delivered short-hairpin RNA

Lentiviral vectors, targeting OPA1 and Mfn1 and control, were produced in 293T cells as previously described. To generate stable gene knockdown cells, Huh7 cells were transduced with lentiviral vectors obtained from the Erasmus Center for Biomics. Control vector (shCTR) is a short hairpin sequence containing 5 bp mismatches and scrambled sequences not aligning to any known human or mouse gene. Since the vectors also express a puromycin resistance gene, transduced cells were subsequently selected by adding 2.5 µg/ml puromycin (Sigma) in the cell culture medium. After pilot study, the shRNA vectors (supplement table 1) exerting optimal gene knockdown were selected by qPCR with the corresponding primers shown in supplement table 2. The amount of HEV was assessed after 3 days of infectious HEV medium post-infecting shCTR cells and knockdown cells.

Western blot assay

Proteins in cell lysates were heated 5 min at 95 °C followed by loading onto a 10-15% sodium dodecyl sulphate-polyacrylamide gel (SDS-PAGE) and separated by electrophoresis. After 90 min running at 100 V, proteins were electrophoretically transferred onto a polyvinylidene difluoride (PVDF) membrane (Invitrogen) for 1.5 h with an electric current of 250 mA. Subsequently, the membrane was blocked with blocking buffer. It was followed by incubation with OPA1 (1:1000) antibodies overnight at 4 °C. The membrane was washed 3 times followed by incubation for 1 h with anti-rabbit IRDye-conjugated secondary antibodies (LI-COR Biosciences, Lincoln, USA) (1:5000) at room temperature. Blots were assayed for β-actin content as standardization of sample loading, scanned, and quantified by Odyssey

infrared imaging (Li-COR Biosciences, Lincoln, NE, USA). Results were visualized and quantitated with Odyssey 3.0 software.

Statistical Analysis

Statistical analysis was performed using a nonpaired, nonparametric test (Mann-Whitney test; GraphPad Prism Software). P values of less than 0.05 were considered statistically significant.

Results

HEV infection induces mitochondrial fusion

Up to date, the impact of HEV infection on mitochondrial dynamics is unknown, prompting investigation in this respect. To this end a high concentration stock of HEV particle was prepared by ultracentrifugation of lysed (freeze thawing) HEV infected Huh7 cells, whereas a similar preparation of non-infected Huh-7 cells was used as control. Subsequently were inoculated with resulting preparations for 3 days after which cells were fixed and analyzed by immunofluorescence and confocal microscopy for mitochondrial characteristics. As evident from Figure 1A, these experiments revealed obvious elongated mitochondria in HEV infected cultures, apparently indicating mitochondrial fusion, whereas uninfected cells displayed the canonical rod-shaped mitochondria. This notion was confirmed by morphometric analysis in which the length of induvial mitochondria was directly quantified (Figure 1B) and the changes in mitochondrial shape correlated with production of HEV RNA as analyzed by qRT-PCR (Figure 1C) showing that these effects related to the infectious process. Subsequently, we investigated whether HEV can induce mitochondrial fusion in other types of cells as well and results as obtained with Huh7 cells were observed in the liver cell line HeprG, the canonical HEV infection model A549, and in intestinal CaCo2 cells (Figure 2). We concluded that HEV infection is associated with clear alterations of mitochondrial dynamics and experiments were initiated to dissect the molecular basis of these events.

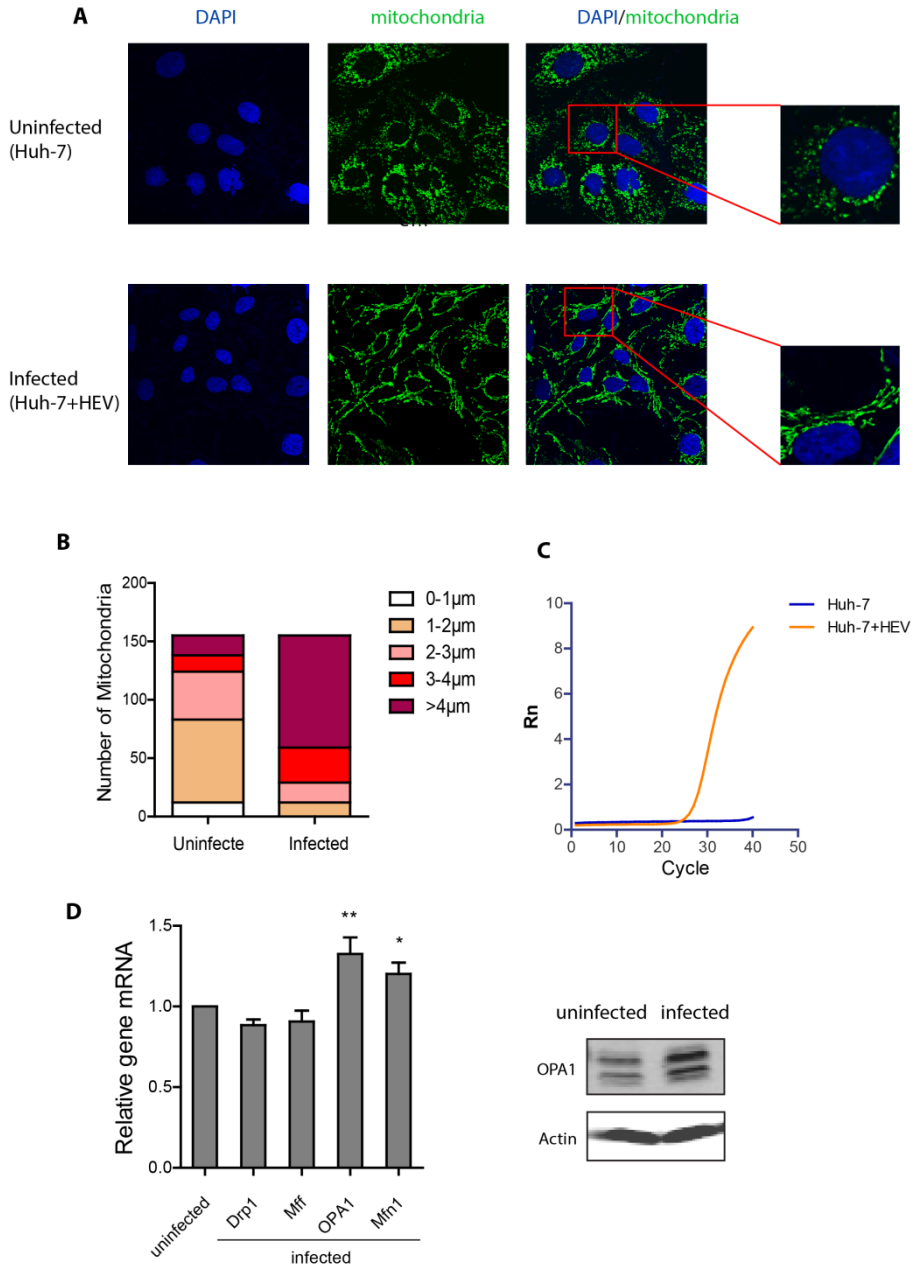

Figure 1. HEV secondary infection induces mitochondrial fusion. Immunofluorescence analysis showing mitochondrial fusion in HEV infected Huh7 cells. Huh7 cells infected with HEV particles obtained from freeze-thawing infected cell pellets (viral copy number, 5,96 × 10^7) or with a preparation obtained from freeze-thawing uninfected Huh7 cell pellets. At 3 days post infection, cells were immunostained with anti-HSP60 (mitochondrial marker) and DAPI (nuclei). Shown confocal images are representative for three independent experiments. (63x oil immersion objective). (B) Quantification of mitochondrial length was performed by imageJ software and is shown in (B). (C) qRT-PCR analysis showed the Ct value of targeting HEV sequence in mock Huh7 cells and HEV infected Huh7 cells. (D) Transcription of 4 mitochondrial dynamics regulatory proteins was assessed by qRT-PCR and the OPA1 expression was measured by Western blot.

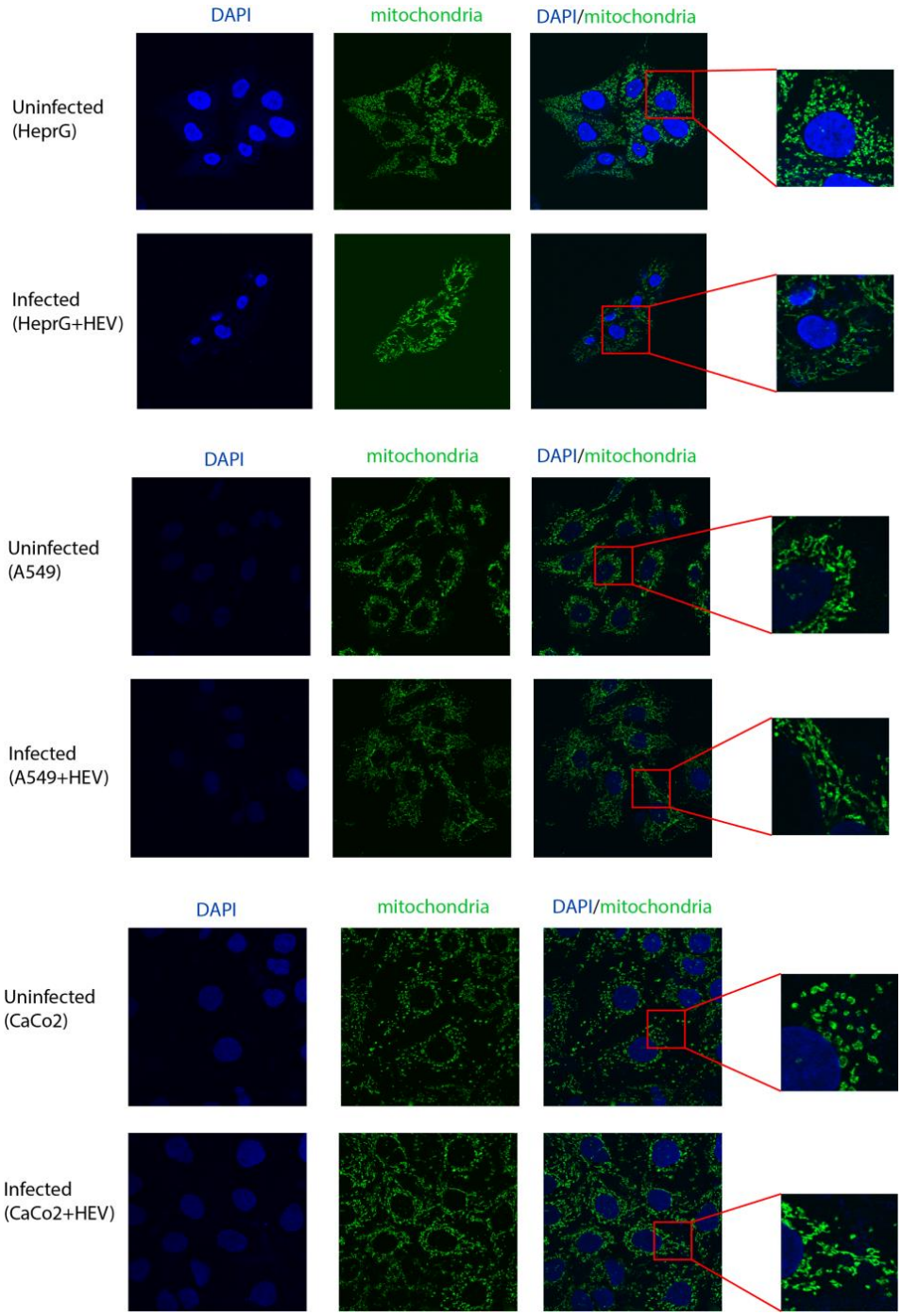

Figure 2. HEV secondary infection induces mitochondrial fusion in a variety of cell types. Hepatocyte HeprG cells, lung cancer cell A549, and intestinal cell CaCo2 were infected with HEV particles obtained from freeze-thawing the cell pellets of infected Huh7 cells. At 3 days post infection, cells were immunostained with anti-HSP60 (mito) and DAPI (nuclei). Shown confocal images are representative for three independent experiments. (63x oil immersion objective).

We next examined whether electroporation of full length of HEV genome in Huh-7 cells also alter mitochondrial dynamics. HEV ORF2 protein (red) and mitochondria (green) were detected three days electroporation of HEV. As shown in Figure 3, HEV infected cells

displayed distinct elongated mitochondria. This confirms that mitochondrial fusion is a consequence of HEV infection.

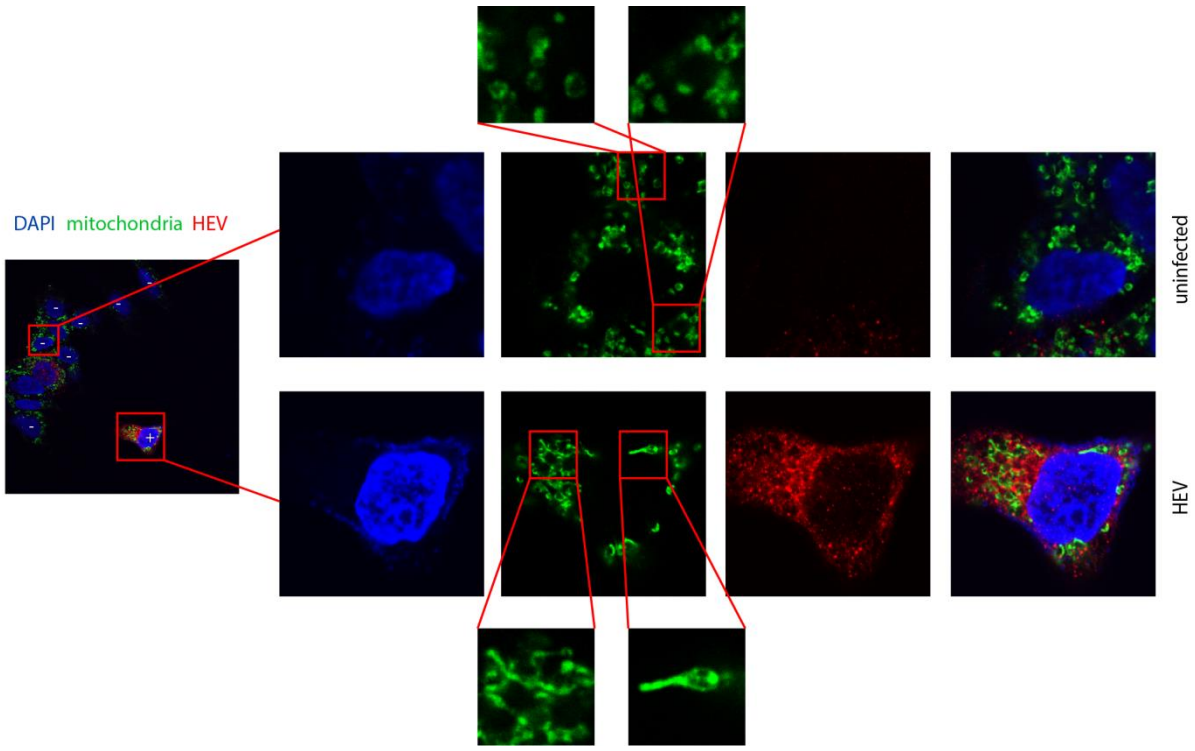

Figure 3. Electroporation of HEV induces mitochondrial fusion. Immunofluorescence staining of viral protein ORF2 (red) and mitochondria (green) in Huh-7 cells upon electroporation of the full-length genomic HEV RNA. DAPI (blue) was applied to visualize nuclei. Shown confocal images are representative for three independent experiments. (63x oil immersion objective).

HEV infection provokes expression of mitochondrial fusion -stimulating proteins

Mitochondrial morphology is subject to dynamic regulation, these organelles constantly fusing and fission, also to meet alterations in metabolic demand or in response to mitochondrial damage. This regulation is exerted by key regulatory proteins that either facilitate mitochondrial fusion or induce their fission [12,13]. Mitochondrial fusion is mediated by the inner membrane fusion protein dynamin-related GTPase (OPA1) and the outer membrane fusion protein Mitofusin1 (Mfn1), whereas fission is brought about by mitochondrial fission factors (Mff) and dynamin-related protein 1 (Drp1). Altered expression of these gene products is thus an obvious explanation for the effects of HEV infection on mitochondrial morphology. Hence, after qRT-PCR analysis was performed to determine alterations in the transcription of these four genes in response to hepatocyte HEV infection (Figure 1D). In line with the evident mitochondrial fusion following HEV infection, we

observed increased levels of OPA1 and Mfn1 transcripts HEV infected cells. No clear effect, however, on the levels of Drp1 and Mff transcripts was observed. The relevance of these observations was supported by experiments in which we analyzed OPA1 expression on the protein level. The Western blot in Figure 1D reveals increased OPA1 protein levels in HEV infected cells. Hence our results show that increased HEV-provoked expression of mitochondrial fusion proteins underlies the effects of HEV infection on mitochondrial morphology [14].

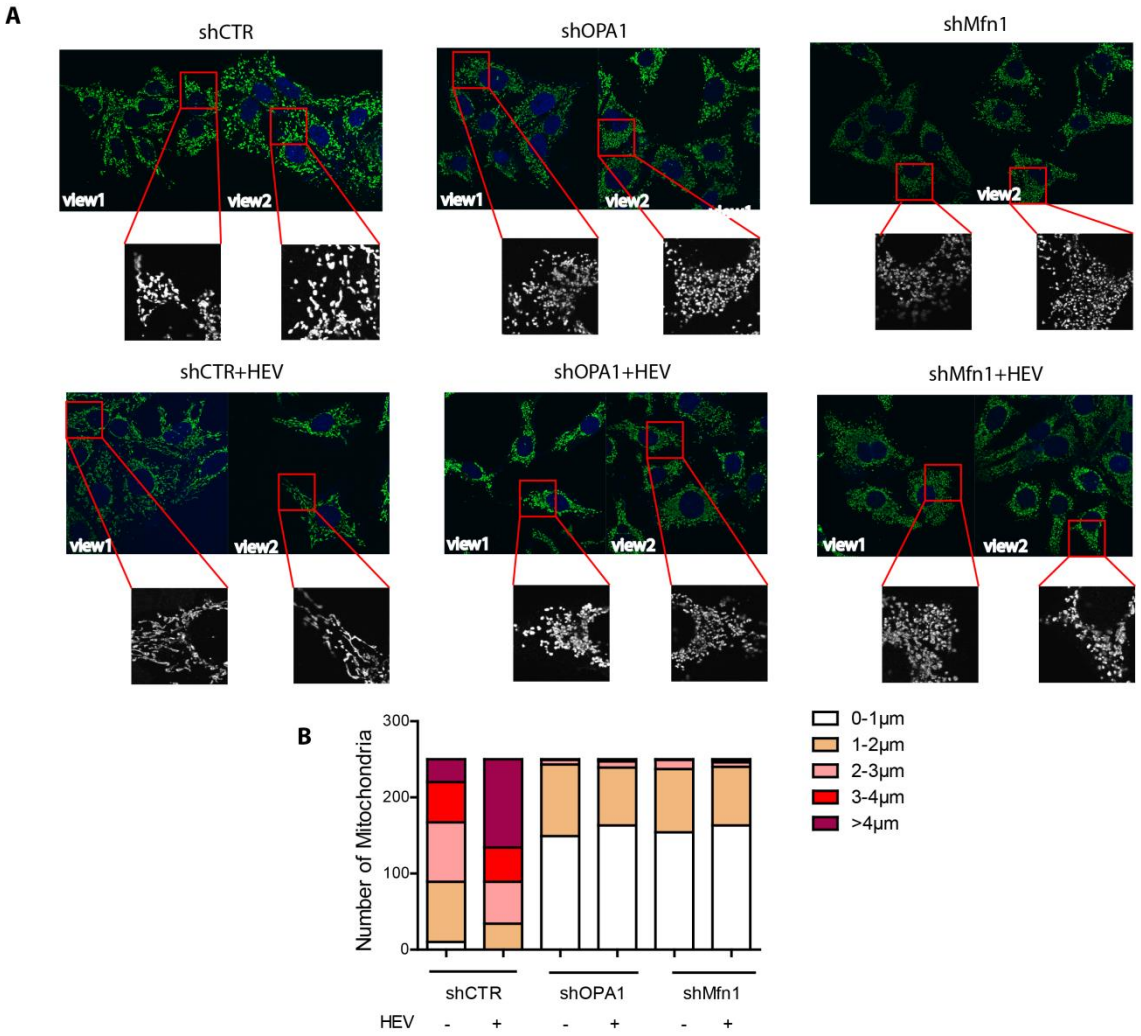

Figure 4. Silencing OPA1 and Mfn1 abrogates HEV-induced mitochondrial fusion. Huh7 cells were transduced with lentiviral vectors targeting OPA1, Mfn1 and control. Upon selection of clones displaying substantial gene silencing, cells were infected with HEV particles. Confocal images showing the inhibition of HEV induced mitochondrial fusion by silencing OPA1 or Mfn1 (A). (B) Quantitative analysis of mitochondrial length in these cultures.

HEV induced mitochondrial fusion facilitates HEV replication

Subsequently we decided to investigate the functional importance of HEV-induced mitochondrial fusion. To this end Huh7 cells were transduced with shRNA-expressing lentivirus either containing a scrambled control sequence or sequences expecting to silence expression of OPA1 and Mfn1. After puromycin selection and expansion of the resulting clones, we found that silencing either OPA1 or Mfn1 induced mitochondrial fragmentation, as revealed by a significant increase in the number of mitochondria per cell and a decrease in mean size per mitochondrion compared to control vector-transduced cells (Figure 4A). The apparent functionality of OPA1 and Mfn1 in our experimental system with respect to the regulation of mitochondrial elongation supports the notion that HEV effects on mitochondrial morphology are mediated by altered expression of these fusion proteins. Definitive proof of this line of thought came from experiments in which the transduced cells were infected with HEV for 3 days. Silencing OPA1 or Mfn1 abolished HEV effects on mitochondrial fusion, whereas the control vector did not have such effects (Figure 4A) and morphometric analysis of mitochondrial length confirmed this interpretation (Figure 4B). Importantly when subsequently the effects of inhibiting of mitochondrial fusion on the HEV infectious process were determined by qRT-PCR, we observed that knockdown of OPA1 and Mfn1 significantly suppressed HEV infection (Figure 5). Thus induction of mitochondrial fusion by HEV facilitates infection.

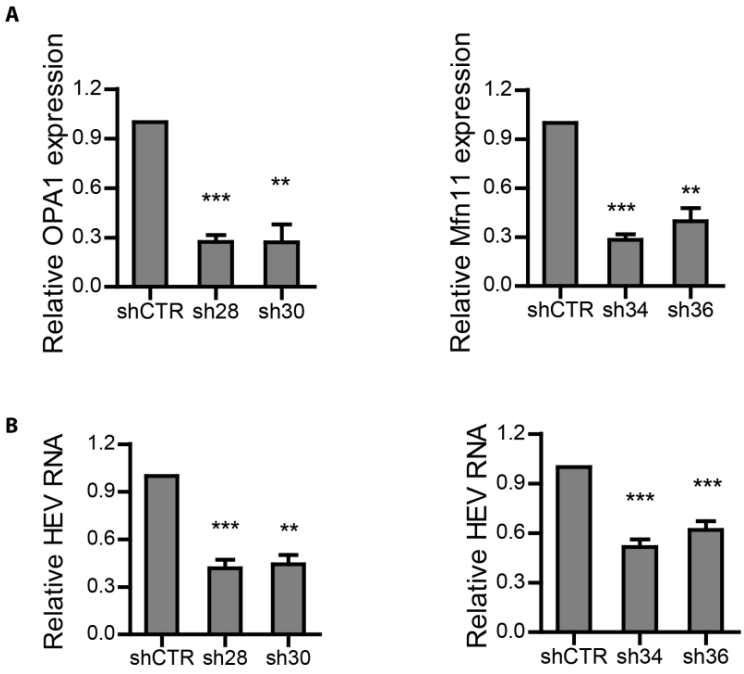

Figure 5. Inhibition of mitochondrial fusion affects HEV infection. Huh-7 cells were transduced with lentiviral vectors targeting OPA1, Mfn1 and control. Upon selection of clones displaying satisfactory knock down, cells were infected with HEV virus. At 3 days post infection, qRT-PCR was performed to establish the resulting HEV mRNA levels.

Disruption of mitochondrial fusion provokes cellular antiviral immune responses

ISGs are the ultimate cell-autonomous antiviral effectors. The mechanisms leading to production of ISGs following viral infection are now partially understood and appear to involve signaling steps at the mitochondrial level, especially relating to MAVS (mitochondrial anti-viral signaling protein). Thus reduced ISG induction following HEV-provoked mitochondrial fusion may constitute explanation of the observed effects of mitochondrial fusion on HEV replication. Interestingly, knockdown of OPA1 or Mfn1 stimulated the expression of a panel of antiviral ISGs during HEV infection and thus diminished IGS transcription may be the relevant cellular target for HEV-provoked mitochondrial fusion (Figure 6).

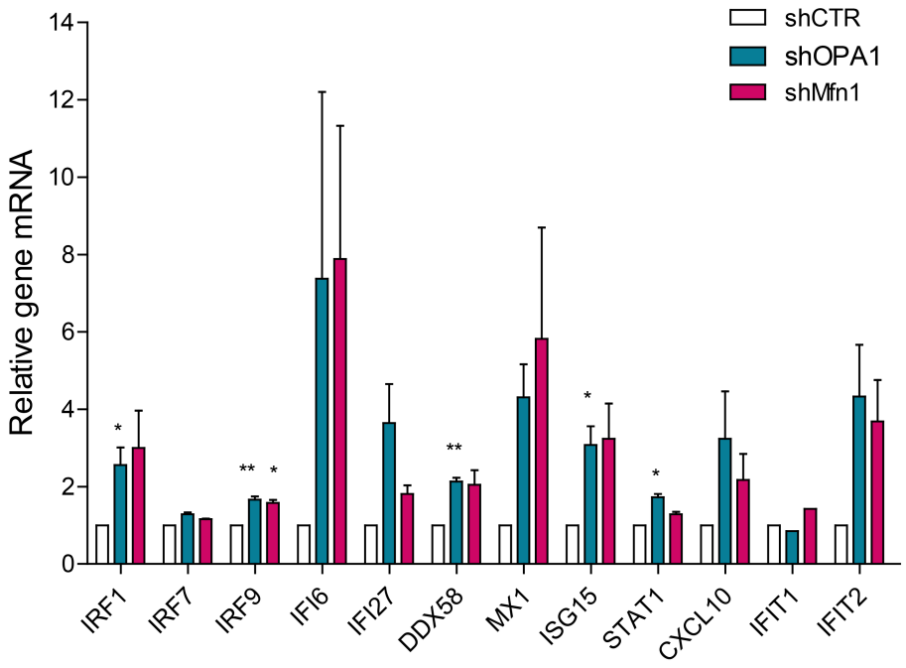

Figure 6. Inhibition of mitochondrial fusion increases antiviral innate immunity. Huh7 cells with OPA1 and Mfn1 silence were infected with HEV particles. The expression of a panel of ISGs was determined by qRT-PCR at 3 days post infection. Data were normalized to basal ISG expression in cells transduced with a scrambled shRNA (white bar, set as 1).

Disruption of mitochondrial fission promotes HEV infection

Mitochondrial fission is initiated by recruitment of cytosolic Drp1 to the mitochondrion and is thus a principal regulator of the mitochondrial fission process [15]. The apparent importance of Drp1 in mitochondrial dynamics raises obvious questions as to the importance of this protein in restraining HEV infection through stimulating cell-autonomous

immunity. As phosphorylation on residue S616 residue of Drp1 by CDK1 seems the main biochemical event provoking translocation of Drp1 to the mitochondria. Thus treatment of cells with CDK1 inhibitors in the context of HEV infection should provide insight into the role of Drp1 in particular and the mitochondrial fission machinery in general with respect to their role in HEV infection. As shown in Figure 7, treatment with CDK1 inhibitor in HEV infected Huh7 cells provoked the appearance of grossly-enlarged mitochondria (Figure 7A), which was accompanied by stimulation of HEV replication and infection (Figure 7B). Thus these results further emphasize the relation between mitochondrial morphology and the HEV infectious process.

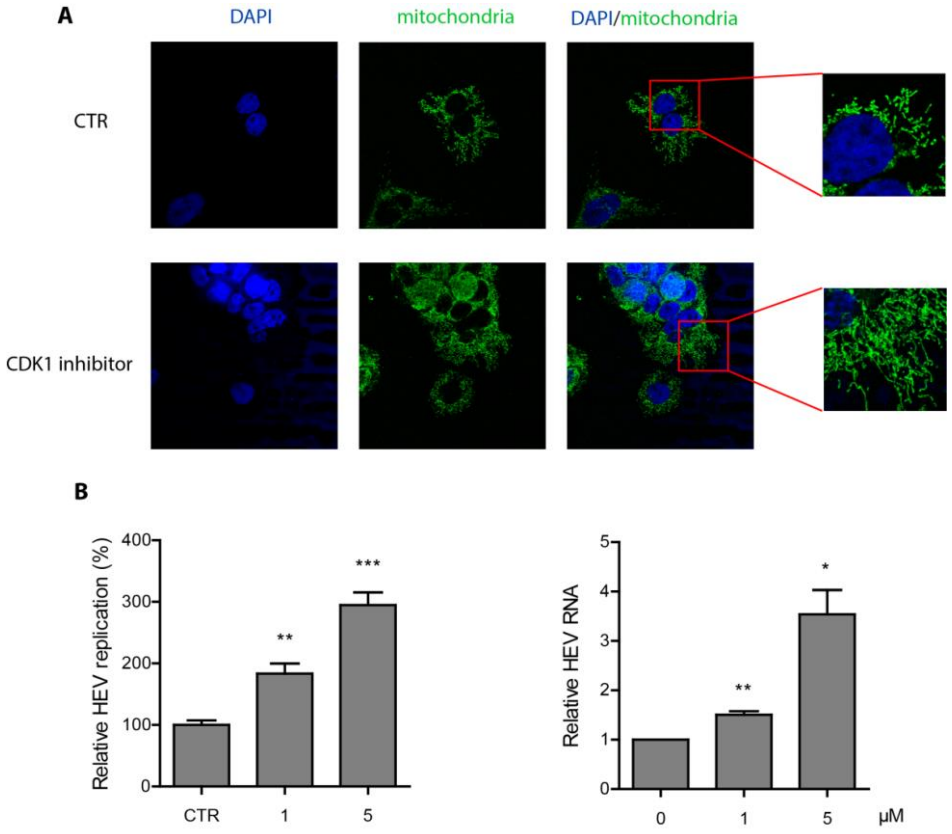

Figure 7. CDK1 inhibition increases concomitantly mitochondrial fusion and HEV infection. (A) Huh7 cells were treated with the CDL1 inhibitor for 2 days and confocal images showing the morphology of mitochondria. (B) Stable infectious HEV cells were directly treated with CDK1 inhibitor for 2 days, HEV mRNA were assessed by qRT-PCR.

The inhibitory effects of impaired mitochondrial fusion on HEV infection are independent of mitochondrial DNA dysfunction

In cells mitochondria constantly fuse and fission to ensure integrity of mitochondrial genome and to ascertain proper functionality of this organelle compartment. Accordingly, OPA1 has important roles in maintaining mitochondrial DNA (mtDNA) stability and integrity [16] and induction of excessive mitochondrial fission in response to interfering with the mitochondrial fusion machinery has been associated with mtDNA instability as evident by reduced mtDNA copy numbers and an increase in the amount of mutant mtDNA [16-19]. It is thus possible that the effects seen with respect to the effects of modulating mitochondrial fusion on HEV replication result from mitochondrial dysfunction per se rather as constituting specific effects on the HEV infectious process. Thus we evaluated the effects of OPA1 knock-down on the quality of the mtDNA. mtDNA polymerases γ (POLG) encoding protein is an essential enzyme for mtDNA replication. We found silence of OPA1 significantly decreased POLG transcription (Figure 8A). In addition, we evaluated transcription of Cytochrome c oxidase I (COX1) proteins, which are encoded by mitochondrial DNA, serving as relatively quantification of mtDNA. The expression of COX1 were evidently reduced in OPA1 silenced cells, in contrast with scramble knockdown cells (Figure 8A). These data suggested that modulating mitochondrial dynamics indeed affected mitochondrial functionality in our model system, with possible effects on the capacity of cells to sustain HEV infection. Hence, we decided to investigate the effect of full mtDNA depletion on HEV infection. To this end Huh7 cells were exposed to a low concentration of ethidium bromide (EB), with is well known to affect mtDNA replication by intercalation of EB into mtDNA genome without affecting nucleus genome. Indeed, when cellular mtDNA levels were measured by transcription of COX1 and ND1 protein of complex I (MTND1) relative to the nuclear-encoded GAPDH by real-time PCR, expression of both mtDNA encoding genes MTND1 and COX1 were evidently diminished following EB treatment (Figure 8C). Intriguingly, upon a subsequent inoculation of significant promotion of HEV infection was observed in mtDNA-depleted cells as compared to control cells (Figure 8B). Similar results were obtained when deoxyribonucleoside analogue dideoxycytidine (ddC) was employed, a compound that destroys mtDNA replication through inhibiting mtDNA polymerase γ (POLG). The ddC pre-treated Huh7 cells were evidently more permissive to HEV infection when compared to

vehicle control-treated cells (Figure 8B). In apparent agreement, when the POLG gene itself was silenced using lentiviral-delivered shRNA and selection for transduced clones using puromycin and experimentation with those clones which displayed most efficient POLG knockdown, it appeared that such knockdown made Huh7 three times more permissive to HEV infection; at least when compared to clones transduced with a scrambled shRNA (Figure 8D). Similarly, when lentiviral-mediated RNAi delivery was used for knockdown the gene PPARGC1A, a gene that encodes peroxisome proliferator-activated receptor c coactivator 1α (PGC-1α), a transcriptional coactivator that positively regulates mitochondrial biogenesis), HEV infection was enhanced (Figure 8E). Thus whereas this experimentation further highlights the link between mitochondrial function and cellular capacity to combat HEV infection, it also demonstrates that overall loss of mitochondrial function upon increasing mitochondrial fission is unlikely to explain to the reduced capacity of HEV to propagate in such cells.

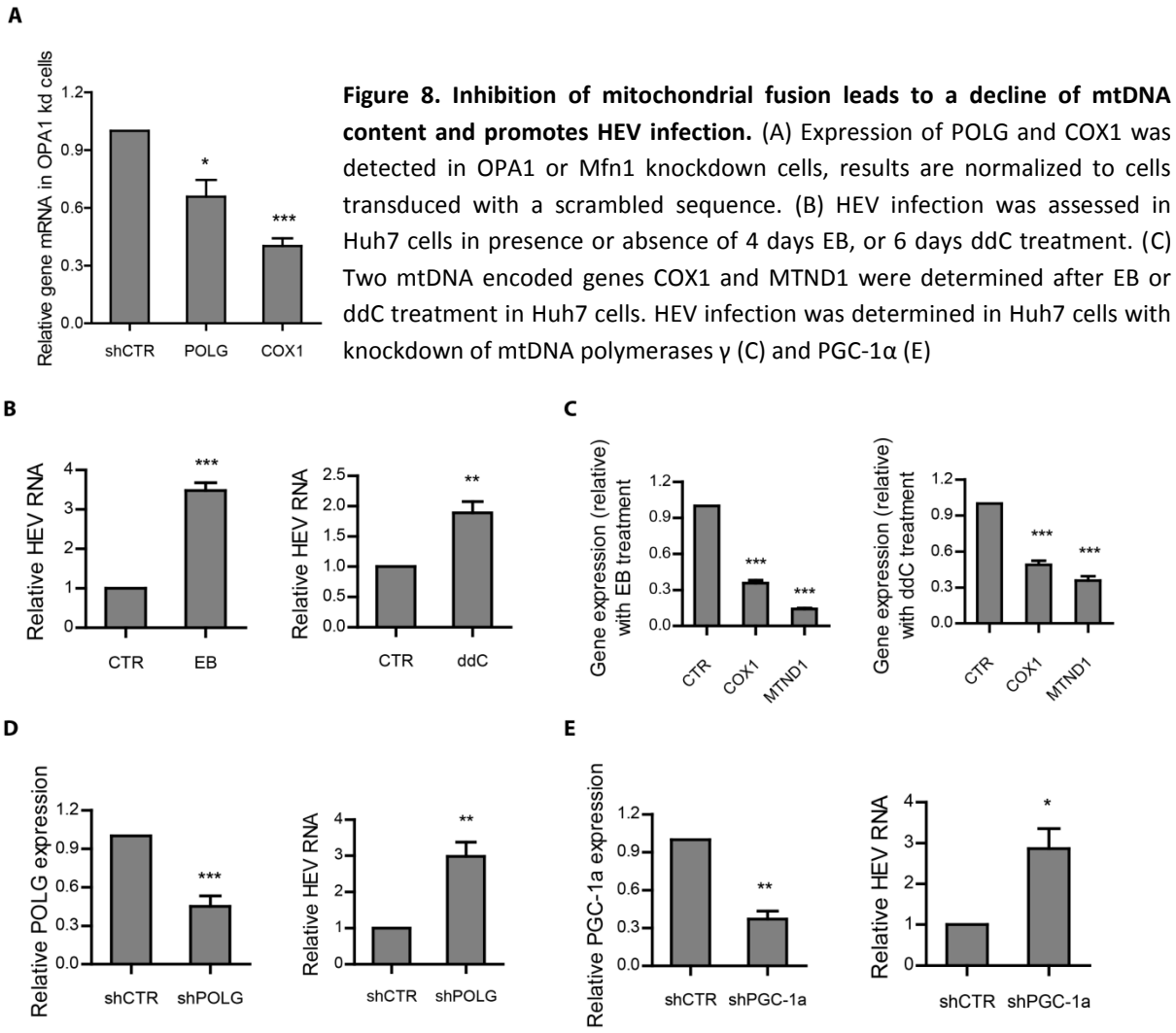

Figure 8. Inhibition of mitochondrial fusion leads to a decline of mtDNA content and promotes HEV infection. (A) Expression of POLG and COX1 was detected in OPA1 or Mfn1 knockdown cells, results are normalized to cells transduced with a scrambled sequence. (B) HEV infection was assessed in Huh7 cells in presence or absence of 4 days EB, or 6 days ddC treatment. (C) Two mtDNA encoded genes COX1 and MTND1 were determined after EB or ddC treatment in Huh7 cells. HEV infection was determined in Huh7 cells with knockdown of mtDNA polymerases γ (C) and PGC-1α (E)

Discussion

The HEV infectious process remains relatively poorly characterized; especially there is a lack of understanding as to the cell biology of the infection and how the host cell machinery is corrupted to perform translation of viral gene products. Generally speaking, however, mitochondrial dynamics have become linked to viral infection in recent years [5]. Here we reveal a novel role for HEV-induced mitochondrial fusion for furthering the HEV life cycle, apparently interfering with cell-autonomous viral immunity. Thus our study has uncovered a previously unrecognized aspect of viral infection, in which the regulation of mitochondrial dynamics is high-jacked to allow the pathogen to escape its constraint by cellular antiviral signaling. It should prove highly interesting to determine whether also other viruses employ likewise strategies. If so, mitochondrial dynamics may become a focus point for the rational design of novel antiviral therapy.

Mitochondrial dynamics were already implicated in immunity. Mitochondria are the location of MAVS, a protein that links intracellular viral detection to the activation of antiviral effectors by stimulating IFN synthesis and NF-κB activation, and the action of MAVS seems to be influenced by mitochondrial fusion and fission [20, 21]. Intriguingly, HBV and HCV also influence mitochondrial dynamics, but these viruses promote mitochondrial fission and mitophagy to attenuate apoptosis and allow persistent infection, whereas HEV-associated mitochondrial fusion appears to serve to suppress cell-autonomous anti-viral immunity [9, 22, 23]. This may relate to chronic properties of HBV and HCV – apparently these viruses are able to co-exist with human immunity and reduction of the infected compartment may be the major evolutionary pressure working on HBV and HCV. In contrast, HEV usually manifests itself as a self-limiting infection, under obvious attack of host immunity and thus more likely to develop cellular immunity subverting strategies. It should thus prove interesting to investigate whether viruses with non-chronic infectious behavior have developed similar strategies to corrupt cellular immunity. In this context it is interesting to note that severe acute respiratory syndrome-coronavirus (SARS) virus, impairs MAVS signaling by mitochondrial fusion via reduction of Drp1 [10]. In conjunction with the findings presented in this study, these data call for further characterization of the interaction of different viruses with mitochondrion-dependent antiviral immunity.

Generally speaking, in cell biology mitochondrial fusion is seen as a pivotal element of the processes that mtDNA stability and copy numbers [17, 19]. A dramatic example of this functionality is found in autosomal dominant optic atrophy (ADOA) patients, who harbor OPA1 and Mfn1 mutations [24]. Furthermore, heterozygous Mfn2 mutations have been associated with mtDNA depletion [16]. Indeed, as recently described in yeast mitochondria by Hori et al., the fusion event facilitates recombination-mediated mtDNA replication and prevents the generation of cells with reduced mtDNA copy number [25]. Accordingly, we observed a decline of mtDNA in the OPA1 or Mfn1 silenced cells. However, a reduction in mtDNA does not relate to the effects seen in this study on HEV replication, as mtDNA per se was associated with increased HEV replication. Thus modulation of intracellular antiviral signaling appears the main effector of the effects of altering mitochondrial dynamics on HEV infection.

Fission of mitochondria is often accompanied by mitophagy and plays an important role in elimination of damaged mitochondrial material [26-28]. Several morphological analyses on the mitochondrial size and its association with mitophagy showed an inverse correlation between mitochondrial elongation and mitophagy [29]. Indeed the process of mitochondrial fission is a key event in the sorting of impaired mitochondria with mutant mtDNA copies from the healthy mitochondrial population [30]. We thus feel it unlikely that HEV-induced mitochondrial fusion is a prelude to mitophagy, but will likely result in an impairment of the disposal of dysfunctional mitochondrial material. The resulting metabolic misbalance and mitochondrial stress may well disrupt antiviral immunity and thus be a factor explaining the effects observed on HEV infection, but obviously further work is essential to substantiate this notion.

Mitochondrial fission and mitophagy are usually accompanied by apoptosis due to accumulated unhealthy pool of mitochondria [31-33],. Numerous of studies have established the positive correlation between fragmented mitochondria and cell apoptosis, evidenced by enhanced cytochrome C release or Bax/Bak activation [12, 13, 31, 32, 34]. Accordingly, mitochondrial fusion has been associated with protection of cells from mitochondrial-dependent apoptosis [13]. HEV mediated mitochondrial fusion might probably dampen cell apoptosis to maintain viral infected cells homeostasis and facilitate HEV persistent infection, this precise role of mitochondrial fusion in adaption to cellar physiological stability

associated with infection deserves further investigation. Interestingly, stimulation of apoptosis was also observed in mitochondrial fission defective cells. Interference of mitochondrial fission induced by HCV infection results in an evident activation of apoptosis signaling, suggesting specific virus can also protect viral infected cells from apoptotic cell death via mitochondrial fission, contrasting to the common notion that apoptosis is accompanied by fission [9].

Viral infection modulates host cell signaling via mitochondrial dynamics to facilitate infection, which might be a primary determinant in the pathogenesis of disease. Mitochondrial fusion induced by HEV appears to play an essential role to help virus evade cell-autonomous immune attack. This finding provides unique insight in to the viral-host interaction respective to HEV infection, and offer novel targets for design of antiviral strategies.

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Supplementary Materials

Supplementary Table 1. shRNA sequences

No.	Gene	ACCESSION	Sequences	Target Sequence
OPA1sh28	OPA1	"NM_130832.1,NM_130831.1,NM_130833.1,NM_130834.1,NM_130835.1,NM_130836.1,NM_130837.1,NM_015560.1"	CCGGCGGGAGTTTGATCTTACCAAACTCGAGTTTGGTAAGATCAAACTCCCGTTTTTG	CGGGAGTTTGATCTTACCAAA
OPA1sh30	OPA1	"NM_130832.1,NM_130831.1,NM_130833.1,NM_130834.1,NM_130835.1,NM_130836.1,NM_130837.1,NM_015560.1"	CCGGCCGGACCTTAGTGAATATAAACTCGAGTTTATATTCACTAAGGTCCGGTTTTTG	CCGGACCTTAGTGAATATAAA
Mfn1sh34	MFN1	NM_033540.2	CCGGGCGGCTTTCCAAGCCTAATATCTCGAGATATTAGGCTTGGAAAGCCGCTTTTTG	GCGGCTTTCCAAGCCTAATAT
Mfn1sh36	MFN1	NM_033540.2	CCGGGCTCAAAGTTGTAAATGCTTTCTCGAGAAAGCATTTACAACTTTGAGCTTTTTG	GCTCAAAGTTGTAAATGCTTT
POLGsh86	POLG	NM_002693.1	CCGGGCAGAGGTGCACAGACTTTATCTCGAGATAAAGTCTGTGCACCTCTGCTTTTTG	GCAGAGGTGCACAGACTTTAT
PGC-1αsh39	PPARGC1A	NM_013261.2	CCGGCCTCCTCATAAAGCCAACCAACTCGAGTTGGTTGGCTTTATGAGGAGGTTTTT	CCTCCTCATAAAGCCAACCAA

Supplementary Table 2. Primer sequences

Gene	Sequences 5’ to 3’
HEV-F	ATTGGCCAGAAGTTGGTTTTCAC
HEV-R	CCGTGGCTATAATTGTGGTCT
DDX58-F	CACCTCAGTTGCTGATGAAGGC
DDX58-R	GTCAGAAGGAAGCACTTGCTACC
ISG15-F	CTCTGAGCATCCTGGTGAGGAA
ISG15-R	AAGGTCAGCCAGAACAGGTCGT
STAT1-F	ATGGCAGTCTGGCGGCTGAATT
STAT1-R	CCAAACCAGGCTGGCACAATTG
IFI27-F	CGTCCTCCATAGCAGCCAAGAT
IFI27-R	ACCCAATGGAGCCCAGGATGAA
IRF1-F	GAGGAGGTGAAAGACCAGAGCA
IRF1-R	TAGCATCTCGGCTGGACTTCGA

IRF9-F	CCACCGAAGTTCCAGGTAACAC
IRF9-R	AGTCTGCTCCAGCAAGTATCGG
IFIT1-F	GCCTTGCTGAAGTGTGGAGGAA
IFIT1-R	ATCCAGGCGATAGGCAGAGATC
IFIT2-F	GGAGCAGATTCTGAGGCTTTGC
IFIT2-R	GGATGAGGCTTCCAGACTCCAA
IFI6-F	TGATGAGCTGGTCTGCGATCCT
IFI6-R	GTAGCCCATCAGGGCACCAATA
IRF7-F	CCACGCTATACCATCTACCTGG
IRF7-R	GCTGCTATCCAGGGAAGACACA
CXCL10-F	GGTGAGAAGAGATGTCTGAATCC
CXCL10-R	GTCCATCCTTGGAAGCACTGCA
MX1-F	GGCTGTTTACCAGACTCCGACA
MX1-R	CACAAAGCCTGGCAGCTCTCTA
OPA1-F	GTGGTTGGAGATCAGAGTGCTG
OPA1-R	GAGGACCTTCACTCAGAGTCAC
Mfn1-F	GGTGAATGAGCGGCTTTCCAAG
Mfn1-R	TCCTCCACCAAGAAATGCAGGC
Drp1-F	GATGCCATAGTTGAAGTGGTGAC
Drp1-R	CCACAAGCATCAGCAAAGTCTGG
Mff-F	CAAGGTTCCAGGCACCGATTTC
Mff-R	GCGACAAAATGCCACGAGCAGA
POLG-F	AGATGGAGAACTTGCGAGCTGC
POLG-R	CACGTCGTTGTAAGGTCCATTGC
PGC-1α-F	CCAAAGGATGCGCTCTCGTTCA
PGC-1α-R	CGGTGTCTGTAGTGGCTTGACT
COX1-F	GATGAGCAGCTTTTCCAGACGAC
COX1-R	AACTGGACACCGAACAGCAGCT
MTND1-F	GGCTATATACAACTACGCAAAGGC
MTND1-R	GGTAGATGTGGCGGGTTTTAGG

CHAPTER 9

Summary and Discussion

Background

HEV was originally identified as an acute and self-limiting infectious disease with in general spontaneous clearance. However, in the last decades, chronic hepatitis and high mortality have been described in various settings, such as in transplant patients and in the immunocompromised. More than 60% of organ transplant recipients infected with HEV develop chronic hepatitis with rapid progression to cirrhosis, and HEV infection is now considered to be an emerging and significant clinical problem. Reduction of immunosupprants is the first-line therapeutic approach for immunocompromised patients with HEV infection, which achieves HEV clearance in approximately 30% of patients, but also leads to side-effect with organ rejection looming large in this respect [1, 2]. Of note, there is no FDA-approved anti-HEV therapy currently available, only interferon-α, ribavirin or a combination have been occasionally used as off-label treatment. Ribavirin is the first line option for anti-viral treatment because of its efficacy with it being well tolerated, safe, and capable of inducing a sustained virological response, as several larger studies have shown. However, the optimal dosage and duration of the treatment still need to be determined and standardized. In addition, both of these medications induce some severe adverse effects. In transplant recipients, elevated risk of organ rejection has been documented with interferon-alpha treatment, and severe hemolytic anemia was observed in patients undergoing ribavirin treatment [3-6]. Taken together, for the management of HEV infection, systematic profiling of immunosuppressive manipulation and its association with HEV infection is of prime importance. In addition, further research aimed at developing effective antiviral treatment by understanding its infection biology and interaction with host cells is urgently required. In this thesis I aimed to address these points.

Immunosuppressive medication in HEV infection

The main clinical challenge is posed by HEV genotype 3 infection in patients receiving orthotopic organ transplantation. Immunosuppressive medication universally used after transplantation to prevent organ rejection appears to be a main risk factor for developing chronic infection. Therefore, dose reduction or even withdrawal of immunosuppressants is considered as the first intervention strategy to achieve viral clearance in these patients. However, clinical evidence has showed different types of immunosuppressants may exert

differential impact on the infection course in patients. Generally speaking, such medication not only inhibits host immunity but also modulates other signaling pathways potentially involved in anti-viral immunity or may even directly affect the viral life cycle. This clinical observation inspired us to explore use of the immunosuppressants on HEV infection in cell culture models (**chapter 2** and **chapter 3**). We found that steroids have no clear influence on HEV replication *in vitro*, while calcineurin and mTOR inhibitors both stimulate HEV replication. The proviral effect of the calcineurin inhibitor, cyclosporine A, is mediated by promotion of HEV replication through inhibition of cyclophilins A and B; another calcineurin inhibitor tacrolimus, only significantly enhances HEV replication at high does. mTOR inhibitors such as rapamycin and everolimus was found to be mediated by blocking an antiviral signaling pathway downstream of mTOR dependent on eIF4E-binding protein 1. A recent study provided *in vivo* evidence of significantly higher HEV RNA levels in patients with chronic HEV receiving an mTOR inhibitor as compared to those receiving calcineurin inhibitors as immunosuppressive regimen [7]. MPA, the active component of mycophenolate mofetil, was found to exert potent *in vitro* anti-HEV activity, mediated by depletion of intracellular GTP pools. Analysis of a limited number of cases suggests that the use of the immunosuppressant mycophenolate mofetil is associated with HEV clearance. Although these findings provide interesting indications on what therapies may be beneficial or detrimental in preventing and treating chronic hepatitis E in transplant patients, they require verification *in vivo*. The current clinical and experimental evidence regarding the key implications of the use of different immunosuppressants in chronic hepatitis E was carefully reviewed in this thesis (**chapter 4**).

Nucleotide synthesis pathways in HEV infection

The proposed mechanism of action for the anti-HEV activity of ribavirin and MPA is the depletion of GTP pools caused by inhibition of IMP dehydrogenase (IMPDH), thus impeding RNA virus replication (**chapter 2** and **chapter 5**). These data suggested viral replication heavily relies on the host cells to supply nucleosides for their propagation. Targeting nucleotide biosynthesis pathways thus represents an attractive strategy for antiviral drug development. Based on that, **in chapter 4**, I comprehensively profiled the role of purine and pyrimidine synthesis pathways in HEV cell culture models. Unexpectedly, targeting the early steps of the purine nucleotide synthesis pathway (before the primary

purine IMP formed) leads to enhancement of HEV replication, indicating these effects may only partly relate to the nucleotide synthesis pathway. It is worth noting that targeting the early stage of purine synthesis results in depletion of ATP and/or GTP pool. Cellular energy metabolism mediated by ATP might be important for the host cells to defend virus infection [8, 9]. Therefore, insufficient ATP level might facilitate HEV infection by escaping from host cellular immunity. However, how the ATP levels regulate virus infection deserves further investigation. Targeting later steps (IMPDH enzyme) results in potent antiviral activity against HEV, an effect apparently relating to purine nucleotide depletion. Inhibition of pyrimidine nucleotide synthesis pathway also powerfully inhibits HEV replication and represents a viable option for antiviral drug development against HEV. Mechanistically, these effects are related to an unconventional interaction with cell-autonomous antiviral immunity dependent on very strong induction of antiviral ISGs. For now the mechanistic details as to inhibitors of nucleotide biosynthesis can induce ISGs remain obscure and need further study. In addition, the concern that the nucleotides depletion restricts viral infection completely relies on activation of antiviral immunity or somehow have direct anti-viral effects remains unknown.

Protein kinase in management of HEV

Protein kinases are principal components of the machineries that orchestrate immune responses against diverse pathogenic entities, including viruses, by subsequent stimulation of specific signal transduction cascades. Numerous pharmacological kinase inhibitors or activators have been broadly implicated kinases as potential avenues for treating various diseases, including cancer, inflammation, diabetes and viral infections [10, 11]. Thus, in **chapter 7**, we comprehensively profile kinase-mediated cascades in cell–autonomous antiviral immunity starting from screening a library of pharmacological kinase inhibitors on the Huh7-based HEV replication cell model. We identified protein kinase C alpha (PKCα) as an important anti-HEV mediator. Thus, our results defined PKCα as a novel antiviral element and machinery against HEV infection. The anti-HEV activity of such inhibitors is fully independent of its downstream NF-κB or AP-1 pathways. These results thus provide a valuable novel target for antiviral therapy, although it remains to be established what mechanisms mediate antiviral activity in this respect, and I call for such investigations to be pursued.

Outlook of HEV-mitochondria interaction

The understanding of HEV biology is still limited despite that decades of research have uncovered several of its characteristics and achieved huge progress with respect to controlling HEV pathogenesis. Virus specific treatment for HEV is not available yet. Further efforts on exploring viral-host interaction, such as in the novel and interesting field of mitochondrial dynamics and its relation to viral infections and disease pathogenesis will possibly extend our knowledge here and aid finding new targets potentially useful to control HEV activity. My primary study, presented in this thesis, shows that HEV-mediated alteration of mitochondrial dynamics is a major determinant of viral infection and sheds light on this novel aspect of HEV infection. I show that HEV exploits mitochondrial dynamics to stimulate viral propagation (**Chapter 8**). However, the precise molecular mechanisms underlying the role of mitochondrial dynamics in HEV infection still remain to be comprehensively described. Although still relatively preliminary, my novel finding describing the association between HEV and host cell organelles opens a great possibility to understand to finally obtain intricate insight into HEV biology and demonstrates that mitochondrial quality control might be a promising therapeutic target in the quest to combat HEV infection and associated liver disease pathogenesis.

I envision that in the future, two dimensions of the relation between mitochondrial function and HEV infection are promising areas for further investigation. One is based on mitochondrial features, including mitochondrial DNA, mitochondrial proteins, mitochondrial membrane potential, as well as mitochondrial dynamics and should establish whether and how these elements interact with HEV infection. The other dimension is cellular signaling regulated by mitochondria, including ATP production, mitophagy, apoptosis, and innate immunity. The role of such mitochondrially-mediated signaling pathways in HEV infection remains to be understood. The complicating point in exploring the mechanisms of HEV effects on mitochondrially-mediated signaling is that these two dimensions may function in conjunction, cross-effecting one dimension with the other. For example, HCV has been demonstrated to induce cell apoptosis; however, the activation of mitochondrial fission protein Drp1 by HCV infection has an important role in preventing apoptosis, suggesting that evolutionary pressure on the virus to prevent cellular apoptosis phenomena but that the resulting effect is not perfect. Thus we should take notion when draw conclusion on the

association between HCV induced mitochondrial fission and HCV induced cell apoptosis. Also taking HCV as a model, HCV infection was previously found to increase ATP production, later, novel findings revealed that inhibition of HCV induced mitochondrial fission led to a decline in ATP level, suggesting that the ATP production promotion might be attributed to the mitochondrial fission process, instead of direct HCV-dependent modulation. In our HEV-based cell culture model altered mitochondrial dynamics not only influenced innate immune response, but also affected mtDNA copy number. The exact contributors to the ultimate effects of HEV on mitochondrial dynamics and vice versa remain to be clarified. Therefore, in future research, the challenge is not only understanding the mitochondrial multifunctionality during viral infection, but also to establish the cascade, and potentially matrix-like or reticular network-like links between individual signaling mediated by virus induced mitochondrial alterations. In addition, virus infection also seems influence mitochondrial responses to changes in environmental stimuli. The main effect of HCV infection on the mitochondrial compartment may be the induction of mitophagy by stimulation of mitochondrial fission, which may serve as a strategy to eliminate damaged and apoptosis-promoting mitochondria and thus facilitates maintaining the compartment harboring persistent viral infection [12]. Intriguingly, however, Hara *et.al.* found that HCV is able to suppress mitophagy induced by CCCP, a widely used reagent for inducing mitophagy [13]. The authors believe this process is a strategy of HCV to sustain the presence of affected mitochondria and that the resulting increased production of reactive oxygen species is involved in the development of HCV progression. Thus questions remain as to the exact link between viral infection and mitochondrial quality control and associated effects on cellular metabolism. Addressing these questions is a challenge that urgently prompts future research.

Final remarks

- Both cell culture based research and clinical evidences suggest that different immunosuppressive regimens can differentially affect the infection course of HEV. These observations indicates that immunosupprants can have direct effects on viral replication, apart from influencing antiviral immunity. Experimential evidences deminstrated the immmunosupprants can target their specific host factors to exert pro- or anti- viral effects, This knowledge gained from this study is certainly a valuable reference for the management of immunosuppression in organ transplant recipients infected with HEV. Hopefully, it will also promote the initiation of randomized controlled clinical studies to address these issues in the near future.

- Targeting nucleotide biosynthesis pathways represents an promising strategy for treatment of HEV infection. As host factors, a number of catalytic enzymes involved in de vovo nucleotide synthesis are attractive targets for future drug development.

- Discovery of regulation of mitochondria properties on HEV indeed sparks a novol avenue of combating HEV infection. Despite many issues remain unclear, the alteration of mitochondrial dynamics exploited by HEV infection to their own benefit can be implicated as a potential therapeutic target against HEV infection. Moreover, The relvence of mitochondrial dynamics and viral infection may help in understanding the pathogenic process. Undoubtelly, it remains important to study multiple mitochondrial functions that associated with mitochondrial dynamics to elucidate the physiological significance of enhanced mitochondrial fusion in the HEV infectious process and disease pathogenesis.

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CHAPTER 10

Dutch Summary

Eén van de verwekkers van virale hepatitis is het hepatitis E virus (HEV). Dit HEV komt via het maag-darm kanaal het bloed binnen en vermenigvuldigd zich dan in de lever. Het verloop van een acute HEV-infectie kent een aantal stadia: van subklinisch, naar acuut en uiteindelijk fulminant. Hepatitis E, dat in toenemende mate wordt gezien als een “public health concern”, heeft een mortaliteit van 0.2 – 1.0 %, maar kan bij zwangere vrouwen in het laatste trimester van hun zwangerschap oplopen tot een mortaliteit van 20-25%. Daarnaast is er zorg over het gevaar van het virus voor andere groepen, met name voor transplantatiepatiënten. Zulke patiënten krijgen immuunsysteem onderdrukkende medicatie voorgeschreven die potentieel de weerstand tegen HEV zouden kunnen verminderen. Orgaan transplantatie gaat gepaard met het gebruik van immunosuppressiva. Immers, anders wordt het getransplanteerde orgaan afgestoten. Verschillende immunosuppressiva werken mechanistisch op andere wijze; het is dus goed voorstelbaar dat sommige immunosuppressiva een directe interactie met de levenscyclus van het HEV en het virus direct remmen, terwijl andere immunosuppressiva niet zo’n interactie zouden hebben. Uiteraard zou het gebruik van een immunosuppressief regime dat tegelijkertijd ook virusreplicatie remt de voorkeur moeten hebben voor patiënten met een verhoogd risico op het ontwikkelen van hepatitis E. In **hoofdstuk 3** en **hoofdstuk 4** ga ik op zoek naar zulke immunosuppresiva. Ik vond dat verschillende immunosuppressiva inderdaad ander interactie met de HEV levens cyclus hadden. Sommige, in het bijzonder remmers van mTOR (een moleculair element dat informatie van de voedingsstaat van de cel integreert met de informatie gegeven door immunostimulatoire hormonen) leken HEV infectie te bevorderen (mTOR lijkt dan ook een element van de verdediging van de cel tegen HEV). Andere immunosuppresiva, zoals steroiden, hadden geen effect, maar mycofenolzuur (een potent middel om orgaanafstoting te voorkomen) remde juist HEV infectie. Dit laatste middel lijkt dan ook aangewezen bij patiënten met een verhoogd risico op hepatitis E

In lijn met deze resultaten vond ik inderdaad bleek uit een systematisch door mij uitgevoerd literatuuronderzoek (**hoofdstuk 4**) met betrekking tot de vatbaarheid van transplantatiepatiënten voor HEV infectie dat zo’n twaalf procent van deze patiënten antistoffen voor HEV in haar bloed had. Bovendien was in zo’n twee procent der patiënten ook viraal RNA aanwezig. In deze laatste groep ontwikkelde 65 % van de patiënten een chronische infectie. Ik concludeerde dat hepatitis E een groot probleem bij orgaan

transplantatie. Naast modulatie van immuunsuppressie is ook direct ook direct antivirale therapie een mogelijke therapeutische optie. Inderdaad beschrijf ik in **hoofdstuk 5** dat het antivirale middel ribavirine een sterke werking tegen het HEV heeft, dat dit antivirale effect nog verder versterkt kan worden door co-therapie met interferon en dat het therapeutisch effects van ribavirine met betrekking tot HEV infectie ligt in haar modulatie van de de novo synthese van nucleinezuren. In **hoofdstuk 6** karakteriseer ik de relatie tussen het cellulaire nucleinezuurmetabolisme en HEV infectie in detail en genereer ik dus nog meer inzich hoe ribavirine HEV kan bestrijden. Ribavirine heeft echter veel bijwerkingen en andere medicatie zou betere eigenschappen kunnen hebben. In praktijk zijn zogenaamde kinaseremmers vaak erg nuttig om ziekte te bestijden. In **hoofdstuk 7** voer ik een zoektocht uit naar kinaseremmers die HEV infectie kunnen bestrijden. Heel onverwacht vond ik dat zogenaamde PKC remmers juist virusinfectie bevorderden. Niet alleen tonen deze data aan dat zulke PKC remmers niet nuttig zijnbij het behandelen van HEV infectie, ze laten ook zien dat PKC een niet eerder onderkende rol heeft in onze virale afweer. Het moduleren van PKC activiteit zou dus een mogelijkheid bieden om mensen te beschermen tegen bekende en nieuwe virussen. In het laatste experimentele hoofdstuk, **hoofdstuk 8** karakteriseer ik tenslotte de interactie tussen de mitochondria (de energiefabriekjes van de cel) en het HEV. Ik laat zien dat het HEV virus fusie tussen mitochondrien teweeg brengt en dat deze fusie noodzakelijk is voor virale infectie, door het saboteren van antivirale responsen in levercellen. Op termijn kan deze observatie ook leiden tot nieuwe medicatie om virale infectie te bestrijden.

In het laatste hoofdstuk (**hoofdstuk 9**) van dit proefschrift tenslotte, probeer ik alle informatie die ik heb vergaard gedurende mijn promotieonderzoek te integreren en te duiden aan hand van de reeds bestaande biomedische literatuur. Ik concludeer dat de biologie van HEV nog vele onbegrepen aspect heeft en dat kennis van deze aspecten belangrijke mogelijkheden biedt voor het ontwikkelen van nieuwe medicatie. . Samen hoop ik dat mijn studies een nieuwe bijdrage hebben in de strijd der mensheid tegen hepatitis E

CHAPTER 11

Appendix

Acknowledgements

List of publications

PhD portfolio

About the author

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List of publications

1. **Yijin Wang**, Xinying Zhou, Yannick Debing, Kan Chen, Luc J.W. Van Der Laan, Johan Neyts, Harry L.A. Janssen, Herold J. Metselaar, Maikel P. Peppelenbosch, Qiuwei Pan. Calcineurin inhibitors stimulate and mycophenolic acid inhibits replication of hepatitis E virus. *Gastroenterology. 2014.*

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PhD portfolio

Name PhD Student	Yijin Wang
Erasmus MC Department	Gastroenterology and Hepatology
PhD period	November 2012-September 2016
Date of Birth:	March 15th 1988
Promoter	Prof. dr. M. P. Peppelenbosch
Co-promotor	Dr. Qiuwei Pan

General Courses

2012 the workshop on photoshop and illustrator CS5

2013 Biomedical English Writing

2014 the virology course and symposium

2016 Confocal microscopy

Oral presentations

2014, Dutch Experimental Gastroenterology and Hepatology Meeting, Netherlands

2014, European Association for the Study of the Liver (EASL), London, United Kingdom

2016, Dutch Experimental Gastroenterology and Hepatology Meeting, Netherlands

Poster presentations

2014, The 20th Annual Molmed Day, Molecular Medicine Postgraduate School, Rotterdam, The Netherlands

2015, The 19th Annual Molmed Day, Molecular Medicine Postgraduate School, Rotterdam, The Netherlands

2015, Dutch Experimental Gastroenterology and Hepatology Meeting, Netherlands

2015, European Association for the Study of the Liver (EASL), Austria

2016, Dutch Experimental Gastroenterology and Hepatology Meeting, Netherlands

2016, European Association for the Study of the Liver (EASL), Spain

Scholarships and prizes

2012, China scholarship Council (CSC) Scholarship (File No. 201207720007)

2014, Academic Young Investigator Travel Awards. The 49th European Association for the Study of the Liver (EASL) (€650)

Grant

2014, Erasmus MC Grants. (Qiuwei Pan, **Yijin Wang**, Dave Sprengers, Bart L. Haagmans, Birgit Koch, Marusela Oliveras).(€50000)

About the author

Yijin Wang, born in Xuzhou, Jiangsu Province, China, on 15th March 1988. She grew up and attended elementary, middle and high school at her hometown, one of the Euro-Asian continental bridges for China and among its six-largest central cities.

In 2006, she moved to Nanjing, the capital of Jiangsu province to study biotechnology in Nanjing medical university for her Bachelor degree. She did her undergraduate internship at Nanjing military region Center for Disease Control and Prevention with research on establish of the cell line of Herpes Simplex Virus 1(HSV1) by the application of genetic engineering technology. After graduation, in 2010, to pursue further high quality education, she came to Holland, which country has a first-class educational facilities and advanced educational philosophy, to start her Master in Wageningen university and research center, specialised medical biotechnology. The advanced education ideas and flexible teaching methods in Holland university favoured her to learn to absorb biological knowledge and skills into researching with the scientific attitude and spirit. She did her thesis research in laboratorial Of Toxicology, Wageningen university by researching the expression of resistin gene in human macrophages and identification of a correlation with the effects of troglitazone (TZDs) on expression of resistin in rodent models. In her last half year of Master, she was recommended by her promoter Ivonne Rietjens to do internship at laboratorial Of Medicinal Chemistry & Pharmacy, University of Illinois at Chicago, USA. Under Supervision of Judy Bolton, she did excellent work on measurement of the selective estrogen receptor modulators (SERMs) on estrogen-induced malignant transformation in human mammary epithelial cells (MCF-10A) and published paper as co-author.

After got the Master degree, she moved back to Holland with sentimentally attached to the country. She was recruited by her promoter Prof. Maikel Peppelenbosch and co-promoter Dr. Qiuwei Pan and initiated her PhD research on Hepatitis E virus at the department of Gastroenterology and Hepatology, Erasmus Medical Center, Rotterdam. She was concentrated on development of the anti-HEV therapies on the basis of understanding viral-host interaction. Specially, she devoted to projects, including (i) The mechanism of action of different immunosuppressants on HEV infection; (ii) development of potential

anti-HEV strategy by profiling nucleotide *de novo* synthesis pathways; (iii) how the HEV modulate mitochondrial dynamics to facilitate its persist infection. In addition to a list of publications, her discovery of potent antiviral activity by plants led to a Erasmus MC grant and harbour promising candidate for economical, less side-effect, complete anti-HEV therapy.

Currently, she has settled up her job by top-level talents project at department of Pathology and Hepatology, 302 military hospital of China, the largest infectious and liver disease hospital in Chinese military. She aimed at further exploring potential anti-viral strategies and expend her research field to fatty liver and liver tumour, meanwhile, expecting achieve medicine translation by use of abundant source of patients samples in addition to fundamental research.